

**Factors affecting transmission of trypanosomes  
through tsetse flies**

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## **Declaration**

**I declare that the research described within this thesis is my own work  
and that this thesis is my own composition**

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## Abstract

The maintenance of human sleeping sickness and nagana across sub-Saharan Africa depends on cyclical transmission of trypanosomes through tsetse flies. Infection rates in tsetse are normally very low as most parasites ingested with a bloodmeal die in the fly gut. Infections which successfully establish in the fly midgut may subsequently mature into mammalian infective trypanosomes in the salivary glands. However, these processes are not automatic and involve tsetse, symbiont, trypanosome and environmental factors.

Previous work showed that the symbiotic bacterium *Sodalis glossinidius* was involved in susceptibility to trypanosome infection. Streptozotocin (a toxic analogue of the bacterium's main food source) has been recently shown to decrease trypanosome infection rates in the offspring of treated tsetse. In the present work streptozotocin did remove *S. glossinidius* from the offspring of treated flies but it was not possible to generate a line of tsetse free from *S. glossinidius* infection.

Other potential factors involved in acquisition of trypanosome infection were then examined. A range of antioxidants or cyclic GMP were shown to prevent trypanosome death in the tsetse midgut. The process was shown to be independent of protein synthesis as D-cysteine (an unphysiological isomer of L-cysteine) also enhanced midgut infection rates. Further experiments showed that cGMP could significantly inhibit trypanosome death when fed up to 96 h post-infection, whereas antioxidants only functioned for 48 h post-infection. Moreover it was found that maturation of established midgut infections could be regulated by environmental stimuli as well as by antioxidants. Cold shock of infected flies as well as addition of L-cysteine but not D-cysteine to the bloodmeal resulted in significant increases in maturation rates, while nitric oxide synthase inhibitors reduced maturation rates.

It is concluded that reactive oxygen species play a major role in killing trypanosomes entering the tsetse midgut and that cysteine containing proteins and/or nitric oxide are essential for differentiation of established midgut infections into mammalian infective salivary gland infections.

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## Abbreviations used

8-Br-cGMP	8-Bromo-cyclic guanosine monophosphate
8-Br-cAMP	8-Bromo-cyclic adenosine monophosphate
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BUT 135	Butaba 135
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
CNS	Central nervous system
CI	Cytoplasmic incompatibility
CRAM	Cysteine-rich repetitive acidic transmembrane
DDT	Dichlorodiphenyl trichloroethylene
DFMO	Difluoromethylornithine
DO	Dyphasi Obwang
EDTA	Ethylenediaminetetraacetic acid
GSH	Glutathione
GSSG	Glutathione disulphide
GTP	Guanosine triphosphate
GUT 3.1	Gutat 3.1
iNOS	Inducible nitric oxide synthase
MG	Midgut infection rate
MMI	Mitsuhashi and Maramorosch medium
mRNA	Messenger ribose nucleic acid
NAC	N-acetyl-cysteine
NAG	N-acetyl-glucosamine
NAME	N-nitro-arginine-methyl-ester
NO	Nitric oxide
NOS	Nitric oxide synthase

PM	Peritrophic membrane
ppf	Pupae per female
ppm	Parts per million
ROS	Reactive oxygen species
SELDI	Surface enhanced laser desorption ionization mass spectrometry
SIF	Stumpy induction factor
SIT	Sterile insect technique
<i>SRA</i>	Serum resistance associated protein
TI	Transmission of infectivity
VSG	Variable surface glycoprotein
wsp	Wolbachia surface protein

# **Chapter 1**

## **General introduction**



## 1.1 Introduction

Disease caused by African trypanosomes is widespread across much of sub-Saharan Africa. A protozoan parasite called the trypanosome (Order: Kinetoplastida; Family: Trypanosomatidae; Genus: *Trypanosoma*) is responsible for causing sleeping sickness in humans and nagana in domestic animals particularly cattle. World Health Organisation figures suggest that 60 million people inhabit areas affected by sleeping sickness and at present it is estimated that there are around 400, 000 cases presented each year, resulting in about 100, 000 deaths (Dumas, 2001; Kabayo, 2002; Welburn and Odiit, 2002).

Nagana caused by several species of trypanosomes pathogenic to cattle has rendered a vast area of sub-Saharan Africa off limits to productive farming.

African trypanosomes are transferred between mammalian hosts by the bite of the tsetse fly. However, in the wild very few tsetse are found with transmissible infections (often less than 1%) as most flies are refractory to infection (Okoth and Kapaata, 1986). This is not due to lack of exposure to an infected bloodmeal as pupae collected from the wild and fed on emergence on an infected host do not show increased infection rates (Harley and Wilson, 1968; Harley, 1971). After ingesting an infective bloodmeal, trypanosomes of different species undergo cycles of development of varying complexity within a susceptible tsetse fly before mammalian infective metacyclics are produced. After transfer into the mammal, the trypanosomes rapidly proliferate in the blood and lymph, waves of parasitaemia follow and eventually trypanosomes invade the central nervous system (CNS). Two species of trypanosomes, *Trypanosoma brucei rhodesiense* and *Trypanosoma brucei gambiense*, cause disease in humans; both are fatal if not treated (Welburn *et al.*, 2001a). Animal trypanosomiasis is widespread across much of sub-Saharan Africa; nagana leads to loss of fitness of cattle, resulting in the reduction in the meat and milk yields and the ploughing capacity of infected animals.

Taken together the effect of trypanosomiasis on the loss of agricultural production and the cost of dealing with the problems arising from human and animal disease (trypanocidal drugs and control programs) totals \$4.5 billion a year (Kabayo, 2002). The

appearance of trypanosomiasis in an area can lead to a forced exit of its endemic population, particularly if human infective forms are involved. Of the 165 million cattle within sub-Saharan Africa, only 10 million are found within tsetse infested areas (Hursey and Slingenbergh, 1995). To compound the problem the most fertile land is often found within these regions and the only cattle found within these areas are native breeds which although tolerant to trypanosome infection, produce less meat and milk compared to exotic breeds. Programs have been set up to reduce the impact of trypanosome infection, including treatment of infected animals with trypanocidal drugs and reducing the local tsetse population with the use of insecticides and traps. The complete removal of tsetse from the island of Zanzibar using the sterile insect technique has allowed local farmers to import trypanosusceptible cattle into the area and it has been reported that production of both milk and beef have substantially risen as a result of this program (Vreysen *et al.*, 2000; Kabayo, 2002).

### **1.1.1 The Kinetoplastida**

Members of the Kinetoplastida cause disease in all classes of vertebrates and are responsible for diseases in both the old and new worlds affecting in total some 550 million people. These include the human diseases sleeping sickness, Chagas disease and both visceral and cutaneous diseases caused by various *Leishmania* spp., all transmitted by insect vectors. Sleeping sickness is caused by the parasites *T. b. rhodesiense* and *T. b. gambiense* vectored by various species of tsetse fly in sub-Saharan Africa. Chagas disease occurring in South America is due to infection with *Trypanosoma cruzi*; unlike African trypanosomes, which are transferred to the mammalian host through the oral route, *T. cruzi* is vectored by triatomine bugs through faecal deposits extruded during feeding (Fistein and Chowdhury, 1973). *Leishmania* parasites cause disease in both the old and new world. Vectored by sand flies they are transferred to the mammalian host through the oral route when the insect feeds due to regurgitation of infected gut contents (Rogers *et al.*, 2004). Kala Azar, caused by *Leishmania donovani*, is fatal without

treatment while cutaneous leishmaniasis results in the appearance of ulcers, usually on the exposed parts of the body, particularly the face, arms or legs (Grevelink and Lerner, 1996).

Members of the Kinetoplastida show unusual cellular and biochemical traits and unique mechanisms of gene expression. The biggest difference amongst members of the Kinetoplastida when compared to other eukaryotic cells is the possession of a single mitochondrion called the kinetoplast. Kinetoplast DNA is made up of a network of interlocked circular molecules of two classes consisting of a few dozen maxicircles (about 20 kb each) and several thousand minicircles (1kb each; Ferguson *et al.*, 1994).

Maxicircles encode classical mitochondrial genes, which are modified post-transcriptionally by the insertion and deletion of uridine residues by guide RNAs encoded by the minicircles (Benne, 1989; Simpson, 1990; Harris *et al.*, 1990).

Kinetoplastida are the only organisms known to compartmentalise glycolysis within a cellular organelle; these organelles are called glycosomes and around 65 are found in *T. brucei* spp. (Tetley and Vickerman, 1991). Glycosomes are responsible not only for glycolysis but also for ether-lipid biosynthesis,  $\beta$ -oxidation of fatty acids and purine salvage (Parsons *et al.*, 2001). Glycolytic enzymes are present in high numbers in bloodstream form trypanosomes which rely on glycolysis and substrate-level phosphorylation for the generation of energy; lower levels of glycolytic enzymes are found in insect procyclic forms which rely on cytochrome-mediated respiration to produce ATP (Furuya *et al.*, 2002). The Kinetoplastida contain unique antioxidant pathways, the glutathione-glutathione reductase system found in most other organisms is replaced by trypanothione and trypanothione reductase which provide the intracellular reducing environment (Fairlamb *et al.*, 1985; Krauth-Siegel *et al.*, 2003).

Unusual methods of gene expression found in Kinetoplastida include polycistronic transcription, *trans*-splicing processing of the pre-mRNA, transcription of protein coding genes by RNA polymerase I and extensive mitochondrial mRNA editing. Polycistronic transcription involves the production of several genes linked to the same promoter (Vanhamme and Pays, 1995). Processing of precursor mRNA then occurs with the addition by *trans*-splicing of a 39-nucleotide miniexon methylated cap at the 5' end and

polyadenylation at the 3' end creating monocistronic mRNAs (Sutton and Boothroyd, 1986). In most eukaryotes RNA polymerase I is responsible for the transcription of a large rRNA gene unit while RNA polymerase II is responsible for transcription of mRNA. However, in *T. brucei* both variable surface glycoprotein (VSG) and procyclin, the major surface coats of the bloodstream forms and the procyclic forms respectively, are transcribed by RNA polymerase I (Gunzl *et al.*, 2003).

### 1.1.2 Evolution of the Kinetoplastida

Why so many members of the Kinetoplastida have entered into parasitic lifestyles is unknown, but two hypotheses have been put forward, disagreeing on whether the first host was a vertebrate or an invertebrate. Both theories, however, agree that trypanosomatids originated from free-living bodonid flagellates. The vertebrate first hypothesis proposed by Minchin (1908) suggested that parasitism started in the guts of vertebrates with the parasites eventually moving into the blood system. As evolution continued, invertebrates which fed on blood appeared, allowing the transfer of the parasites between hosts. The insect first hypothesis proposed by Léger (1904) and supported by Baker (1963) assumes that Precambrian invertebrates were parasitised by bodo-like flagellates. Over time, new species of insects and parasites evolved with some insects adapting to haematophagy; soon a digenetic lifestyle was evolved and there was independent appearance of *Leishmania* from promastigote leptomonads and *Trypanosoma* from epimastigote *Blastocrithidia*-like organisms. It is thought that the salivarian African trypanosomes and *T. cruzi* diverged following the separation of Africa and South America during the mid-Cretaceous period around 100 million years ago (Stevens *et al.*, 1999).

### 1.1.3 The African trypanosomes

*Trypanozoon*, *Nannomonas* and *Duttonella* form the genus *Trypanosoma*. *T. b. gambiense* and *T. b. rhodesiense*, *T. brucei brucei*, *Trypanosoma evansi* and *Trypanosoma equiperdum* make up the sub-genus *Trypanozoon*. The *Nannomonas* group contains *Trypanosoma congolense*, *Trypanosoma simiae* and *Trypanosoma godfreyi* while *Trypanosoma vivax* and *T. vivax viennei* make up the *Duttonella* group. Of these *T. b. rhodesiense*, *T. b. gambiense*, *T. b. brucei*, *T. congolense* and *T. vivax* are economically important tsetse transmitted trypanosomes causing nagana that affect mammals in Africa. Generally they are only found in the presence of the tsetse vector; however, mechanical transmission by tabanids (Desquesnes and Dia, 2003) or Stomoxyinae (Mihok *et al.*, 1995) can occur.

#### 1.1.3.1 The *Trypanozoon* group

The two human forms of sleeping sickness are split geographically by the Rift Valley; *T. b. gambiense* is found to the west while *T. b. rhodesiense* occurs to the east. Although first described by Forde (1902) the West African form was named *T. gambiense* by Dutton (1902) while Stephens and Fantham (1910) described *T. rhodesiense*. They were later reclassified as *T. b. gambiense* and *T. b. rhodesiense* respectively. *T. b. gambiense*, *T. b. rhodesiense* and *T. b. brucei* are morphologically indistinguishable from each other; however, their effects on humans are radically different. Infection with *T. b. gambiense* results in a chronic infection lasting many years, however, those infected with *T. b. rhodesiense* progress much quicker, with >80% of deaths occurring within the first six months (Welburn *et al.*, 2001a), *T. b. brucei*, however, is non pathogenic to humans as it is lysed by human serum (Tomlinson *et al.*, 1995; Vanhamme and Pays, 2004).

Thought to be a disease under control in the 1950's, sleeping sickness has returned to areas previously cleared of infection and in the last decade, cases of sleeping sickness have been rising and outbreaks have been reported in previously unaffected regions. In Sudan there have been epidemics of *T. b. gambiense* (Moore and Richer, 2001) and *T. b. rhodesiense* has spread to areas of Uganda without a history of sleeping sickness (Fevre *et al.*, 2001). The first recorded sleeping sickness epidemic occurred during the first 20 years of the 20<sup>th</sup> century with around 250 thousand deaths reported (Fevre *et al.*, 2004). Various control measures were put in place to control the outbreaks of sleeping sickness; these included the clearing of the bush and destruction of game animals. This reduced the tsetse breeding habitats and food sources respectively and resulted in a reduction in the number of sleeping sickness cases. Since the first major epidemic in Uganda and despite much effort by the governments of affected countries, epidemics have recurred which have usually coincided with periods of civil unrest (Hutchinson *et al.*, 2003). The emergence of drug resistance has also played a role in the increasing numbers of sleeping sickness cases (Brun *et al.*, 2001; Maser *et al.*, 2003).

The lifecycle of *T. brucei* is shown in Figure 1.1 while the pathway trypanosomes take through the tsetse fly are shown in Figure 1.2. Once ingested by the tsetse fly, mammalian bloodstream form trypanosomes transform into procyclic forms. This transformation is associated with loss of the bloodstream specific VSG coat and its replacement with procyclin and morphological changes including repositioning and activation of the kinetoplast before re-entry into the proliferative cell cycle.

Most trypanosomes do not survive the encounter with the fly gut and only a small proportion of flies develop a parasite population in the fly midgut. Upon reaching the midgut trypanosomes enter the ectoperitrophic space where they continually divide.



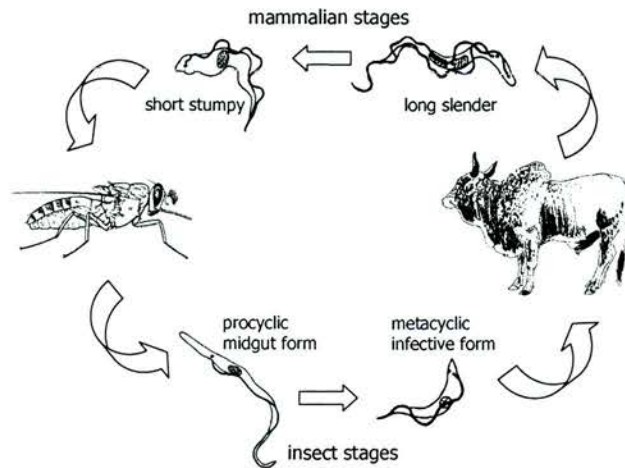


Figure 1.1: Lifecycle of *T. brucei* (kindly provided by Dr Sue Welburn)

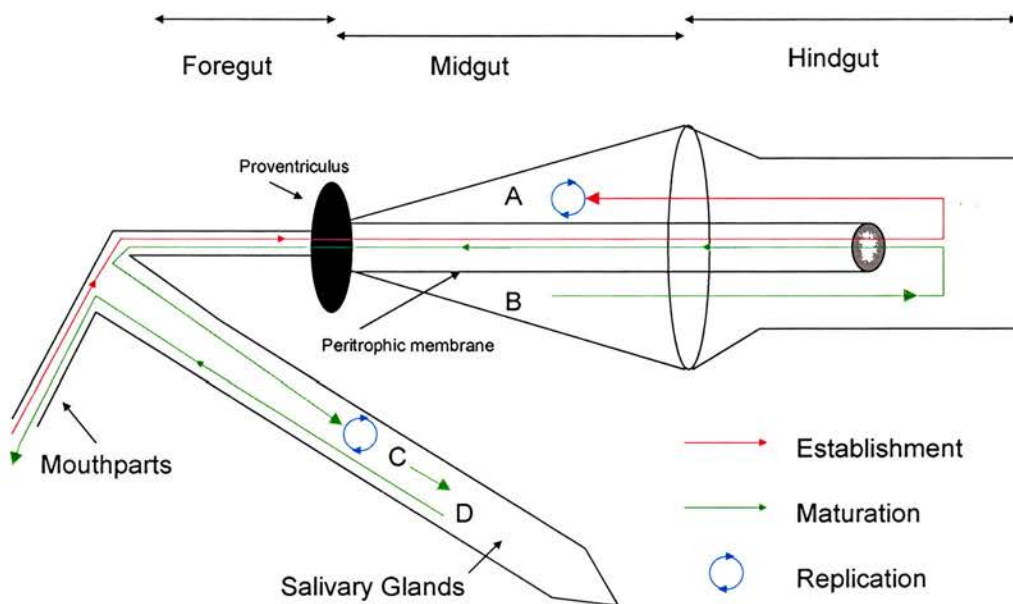


Figure 1.2: Diagrammatic representation of the trypanosome pathway in the tsetse fly. While feeding on an infected host, trypanosomes (*Trypanozoon* and *Nannomonas* groups) enter the fly through the proboscis. After navigating the fore- and mid-guts, trypanosomes enter the ectoperitrophic space (A) via the distal end of the peritrophic membrane in the hind gut. In a susceptible fly they replicate and establish a population of several hundred thousand individuals. In some cases procyclic form trypanosomes begin the journey (B) to the salivary glands (*Trypanozoon* group) where they replicate (C), before finally maturing (D) to form mammalian infective metacyclics. In the *Nannomonas* group maturation occurs in the mouthparts of the tsetse, while the complete developmental cycle of the *Duttonella* group is exclusive to the mouthparts.

Whilst there have been reports of trypanosomes within the ectoperitrophic space within 30 min of infection (Freeman, 1970), recent reports suggest that they do not enter until day six (Gibson and Bailey, 2003). The method of entry into the ectoperitrophic space is controversial and two forms of entry have been proposed; firstly direct penetration of the peritrophic membrane (PM; Freeman 1973; Ellis and Evans, 1977) and secondly entry at the dorsal end of the PM (Maudlin, 1991; Welburn and Maudlin, 1992). Tsetse flies possess a type II PM that is continuously synthesised by the proventriculus, composed of sugars and protein. Its main function is to prevent damage to the midgut epithelium during bloodmeal breakdown (Moloo *et al.*, 1970; Lehane *et al.*, 1996). The first method of entry relies on the trypanosomes penetrating the PM, a method employed by both *Plasmodium* (Huber *et al.*, 1991) and *Leishmania* (Schlein *et al.*, 1991) parasites. Previous results suggested that teneral flies were easier to infect because their PM was not fully formed, while non-teneral flies, which are more refractory to infection, possessed a fully formed PM. However, Welburn and Maudlin, (1992) demonstrated that the addition of D+ glucosamine to the bloodmeal of non-teneral tsetse resulted in infection rates similar to teneral flies fed on the same diet, suggesting that the PM played no part in susceptibility to infection and it was suggested that trypanosomes entered the peritrophic space via the free end of the PM. Within the ectoperitrophic space the trypanosomes multiply, reaching a stable population of around 500, 000 individuals (Welburn and Maudlin, 1997; Van Den Abbeele *et al.*, 1999). Welburn and Maudlin (1997) suggested that this self regulation of numbers by the parasite was carried out by programmed cell death, which would have advantages for both the parasite and the vector, as both fly and trypanosome use proline for their main energy supply. Very few flies, however, show susceptibility to infection with trypanosomes and within three days of bloodmeal ingestion, trypanosomes can be seen dying in the tsetse gut, undergoing a process similar to apoptosis (Welburn *et al.*, 1989). It is thought that lectins produced by the fly bind to the trypanosome inducing cellular death (Maudlin and Welburn, 1987; Welburn *et al.*, 1989). In a susceptible fly, sugars produced by the symbiotic bacterium *Sodalis glossinidius* inhibit the trypanocidal lectins, allowing trypanosomes to establish in the midgut of tsetse (Welburn and Maudlin, 1992).



To complete the lifecycle, midgut form trypanosomes must leave the ectoperitrophic space and migrate to the salivary glands, where they mature to form mammalian infective metacyclics. Little is known about this process although it involves both fly and trypanosome factors (Maudlin and Welburn, 1994; Milligan *et al.*, 1995; Welburn *et al.*, 1995). Once mammalian infective metacyclics have formed, the tsetse can then transmit the infection the next time it feeds.

There are two phases to trypanosome infection in humans, firstly a haemato-lymphatic stage followed by invasion of the CNS. After inoculation by the tsetse fly the parasite transforms into the long slender stage and multiplies at the site of the bite where a chancre forms due to the host's immune response (Barry and Emergy, 1984). Invasion of the circulatory system follows with the parasites multiplying in both the blood and lymph and at this stage non-specific flu-like symptoms are often mis-diagnosed as malaria. The host's immune response mounts a specific immune response to the VSG found on the trypanosome's surface coat, resulting in almost total clearance of the trypanosomes (Vickerman, 1989). However, a small proportion of the trypanosomes change their antigenic coat and the cycle starts over again. This process, called antigenic variation, results in the expression of a new VSG coat which the host immune system does not recognise (Vanhamme *et al.*, 2001). Waves of parasitaemia follow with symptoms including malaise, headache, arthralgia, generalized weakness and weight loss (Kennedy, 2004). During the immune response a short stumpy form of the parasite appears; this non-replicating form is thought to be preadapted to life within the tsetse vector and dies unless taken up by a tsetse fly (Seed and Sechelski, 1989; Turner *et al.*, 1995). However, this hypothesis is controversial as long slender forms can also infect tsetse and recent publications have suggested that the short stumpy is formed to stop parasite numbers killing the mammalian host (Seed and Wenck, 2003).

There then follows invasion of the CNS; in the case of *T. b. gambiense* infections this sometimes does not occur until several years after the initial infection, however, in the case of *T. b. rhodesiense*, invasion can occur in the first few weeks of infection.

Infection by *T. b. rhodesiense* parasites usually results in death within six months of infection whereas in the Gambian form death can take up to five years (Welburn *et al.*,

2001a). Once the CNS is invaded patients usually display the classical sleep related outcomes of disease, including lassitude, distractibility and with spontaneous uncontrollable urges to sleep combined with the reversal of the normal sleep-wake cycle in which daytime somnolence alternates with nocturnal insomnia (Kennedy, 2004). Untreated, the patient progresses to the final stages of the disease, showing symptoms including seizures, severe somnolence, double incontinence, cerebral edema, coma, systemic organ failure and inevitably death.

*T. b. rhodesiense* is a zoonotic disease principally involving transmission of the parasites by the tsetse from animal to human rather than human to human (Welburn *et al.*, 2001a). Large numbers of native game animals carry parasites; however, they do not show overt signs of illness and act as reservoirs of disease. Although mammals other than humans have been found to be infected with Gambian parasites, transmission of the disease by tsetse is usually from human to human although studies have suggested that the domestic pig is a possible reservoir (Mehlitz *et al.*, 1982; Njiokou *et al.*, 2004).

*T. b. brucei* is found throughout sub-Saharan Africa but is non-pathogenic to humans and studies often show it is the least abundant trypanosome found in cattle, when compared to the more pathogenic trypanosomes *T. congolense* and *T. vivax* (Tarimo-Nesbitt *et al.*, 1999). Native cattle show increased resistance towards disease caused by *T. b. brucei* and diagnosis is often not conclusive due to low levels of parasitaemia; however, in dogs, pigs and horses infection often proves fatal.

Human infectivity of *T. b. rhodesiense* is linked to transcription of the serum resistance associated protein (SRA) gene. Experiments by De Greef *et al.* (1989) showed that transcription of a gene encoding a 1.5 kb mRNA was associated with resistance to human serum. PCR analysis, using the SRA gene as a marker showed the presence of the gene in isolates taken from 29 patients all suffering from sleeping sickness; the gene was absent from human serum sensitive isolates taken from cattle (Welburn *et al.*, 2001b). The gene encodes a protein similar to the VSG found on the surface of bloodstream form trypanosomes (De Greef and Hammers, 1994). Recently the lytic factor in human serum has been characterised as apolipoprotein L-I (Vanhamme *et al.*, 2003); SRA inactivates this anti-trypanosomal protein, conveying resistance to human

serum. *T. b. gambiense* parasites do not contain SRA and at present the mechanism(s) conveying resistance to human serum are unknown (De Greef *et al.*, 1992).

*T. evansi* is transmitted both by tsetse flies and by a variety of biting insects including horse and stable flies (Luckins, 1988). Transmission is strictly mechanical with no development occurring within the vector. Found worldwide except for North America and Australia (Luckins, loc. cit.), *T. evansi* causes a wasting disease called surra, with thousands of animals dying each year throughout Africa, Asia and South America; clinical signs of infection are characterised by fever and anaemia, followed by emaciation, oedema and the enlargement of the lymph nodes and spleen, while neurological symptoms may occur late in the infection (Brun *et al.*, 1998).

*T. equiperdum* (Blacklock and Yorke, 1913) causes dourine a venereal disease of horses, which is fatal without treatment (Brun *et al.*, 1998). Although dourine used to be found throughout most of the world, treatment of infected animals and the control of animal movements have reduced its prevalence. Early signs of infection usually consist of swellings and local oedema of the genital organs followed by fever, anaemia and neurological symptoms especially in the later stages of infection (Stephen, 1986).

#### **1.1.3.2 The *Nannomonas* group**

*T. congolense* (Broden, 1904) is found throughout the tsetse belt of sub-Saharan Africa and is the most pathogenic trypanosome to cattle in Africa. *T. congolense* develops in the midgut of the tsetse before maturing in the proboscis of the fly. Cyclical development of *T. congolense* takes between 7 d and 10 d to complete (Dale *et al.*, 1995).

*T. congolense* is sub-divided into three genetically distinct groups based on geographical and vegetational locations; the savannah, forest and kilifi sets represent 90%, 9% and 1% of stocks reported respectively (Bengaly *et al.*, 2002a). The former is also the most pathogenic; experimental infections with the savannah group show higher levels of mortality in both cattle (Bengaly *et al.*, 2002a) and mice (Bengaly *et al.*, 2002b). In

extreme cases death of cattle can occur in a few weeks; however, infected animals can survive for several months to a year. The disease is characterised initially by pyrexia accompanied by loss of appetite and depression. Pyrexia tends to follow waves of trypanosome parasitaemia and in a short time an animal may display a marked loss in condition, alongside other symptoms such as anaemia and respiratory problems.

Both *T. simiae* and *T. godfreyi* are transmitted by tsetse in the same way as *T. congolense*; however, their host range seems to be restricted to suids (Gibson *et al.*, 2001). *T. simiae* is the only trypanosome that shows extreme pathogenicity toward the domestic pig, outcome is usually fatal following a short acute infection. *T. godfreyi* has so far only been isolated from wild tsetse flies; however, it rapidly infects suids producing a chronic occasionally lethal course of infection (Gibson *et al.*, 2001).

#### **1.1.3.3 The *Duttonella* group**

*T. vivax* can be transmitted both cyclically by the tsetse fly and mechanically by other blood sucking insects which act like ‘flying syringes’ (Langley, 1975). As it can be vectored by *Stomoxys* and other biting flies, *T. vivax* spread outwith the tsetse belt of Africa. Outbreaks have been reported in Boliva and Brazil following importation of infected animals (Davila and Silva, 2000). In contrast to members of the *Trypanozoon* and *Nannomonas* groups, *T. vivax* has no midgut stage, with development taking place exclusively within the proboscis of the tsetse. The infective metacyclic forms develop in the hypopharynx, the process taking between 5 d and 13 d depending on temperature (Desowitz and Fairbairn, 1955). *T. vivax* infection is common in cattle in central and eastern parts of Africa where the disease causes relatively mild symptoms and infection with other trypanosomes is considered to be more of a threat. However, in West Africa, the disease is a cause for greater concern and can assume an acute fatal course even in indigenous cattle breeds such as the Zebu (Stephen, 1970). Infected cattle usually die within 5 weeks of infection; symptoms generally include high temperature, lethargy and anaemia.

*T. vivax viennei* is morphologically identical to *T. vivax*, however, *T. v. viennei* appears to have lost the ability to undergo development in the tsetse fly and is instead transmitted mechanically by biting flies.

#### **1.1.4 The trypanosome surface coats**

There are two major surface coats found on trypanosomes, these are VSG and procyclin, present on the surface of bloodstream forms and procyclics respectively. Both VSG (Doering *et al.*, 1989) and procyclin (Field *et al.*, 1991) are attached to the cell surface by glycosylphosphatidylinositol anchors. VSG comprises about 10% of the total protein content of bloodstream trypanosome and completely covers the cell, with each molecule comprising of between 420-460 amino acids (Wang *et al.*, 2003). VSGs show little conservation of primary sequence, however, they contain distinct cysteine residues creating disulphide bridges which may explain why they show conserved tertiary structures (Carrington and Boothroyd, 1996). Waves of parasitemia seen in infected individuals are due to the expression of different VSGs; as the host mounts an antibody response to a particular VSG, a small number of parasites switch the VSG they are expressing, allowing them to escape the host's immune response and continue the infection. Although only one VSG is transcribed at a time it is thought that the trypanosome genome harbours over 1000 different VSG genes (Vanhamme *et al.*, 2001). Transcription of VSG genes is restricted to a set of around 20-40 bloodstream expression sites located at chromosomal telomeres. Only one expression site is active at a time, with the other expression sites being silenced by transcriptional elongation. Switching of the expression of VSG can occur by two separate methods, either by replacement of the gene being transcribed with a new gene which is copied from the genome or by the silencing of the current expression site followed by the transcription of a gene held in another expression site. Both methods of expression have been observed, however, it is thought that the copying of a gene to the current expression site is the predominant form of switching the surface coat (Borst and Ulbert, 2001; Vanhamme *et al.*, 2001). Stumpy

form trypanosomes cannot change their VSG surface coat and will die if they are not taken up by a tsetse fly. Once ingested by a tsetse fly the trypanosome changes its VSG coat to procyclin; expression of procyclin and silencing of VSG is detectable within 30 min of transformation to the procyclic form (Vanhamme *et al.*, 1995). VSG is released by two independent primary mechanisms, initially by hydrolysis and later by proteolysis mediated by a zinc metalloprotease (Ziegelbauer *et al.*, 1993; Gruszyński *et al.*, 2003). Procyclins fall into four classes, three of which contain dipeptide (EP) repeats differing in N terminal domain and the presence or absence of glycosylation. The fourth class which is not glycosylated consists of pentapeptide (GPEET) repeats (Roditi *et al.*, 1998). It is thought that each class of procyclin is present in at least two copies in the trypanosome genome (Ruepp *et al.*, 1997). During transformation in the midgut of the fly GPEET and the four classes of EP are all found on the surface coat of the trypanosome. Although within 24 h GPEET is the major surface component, at around day six members of the EP family predominate (Acosta-Serrano *et al.*, 2001).

### 1.1.5 Tsetse flies

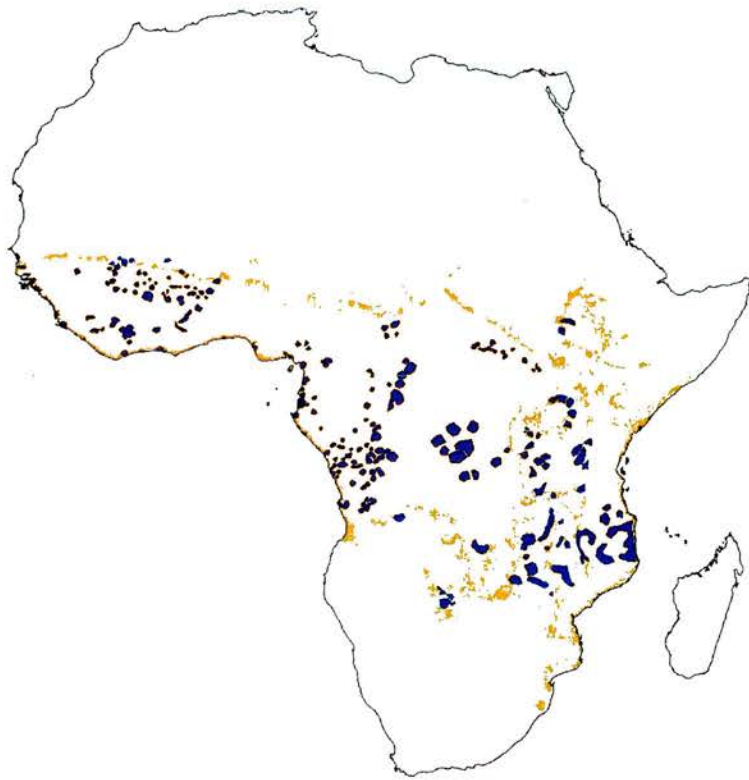
Since Wiedemann (1830) established the genus *Glossina* (Order: Diptera; Family: *Muscidae*), 31 species and sub-species of tsetse flies have been described (Table 1.1; based on Ford and Katando, 1977 and Jordan, 1993). Tsetse have been divided into three sub-genera, these are *Glossina*, *Nemorhina* and the *Austenina*, commonly called the *morsitans*, *palpalis* and *fusca* groups respectively, although some authors have proposed that *G. austeni* should be placed in a group by itself called *Machadomyia* (Chen *et al.*, 1999). Based on both fossil evidence (Cockerall, 1919) and phylogenetic characterisation (Chen *et al.*, 1999; Akman *et al.*, 2003) tsetse flies are thought to have evolved at least 50-80 million years ago originating during the Palaeocene and Oligocene periods.

<b><i>Morsitans</i> group</b>	<b><i>Palpalis</i> group</b>	<b><i>Fusca</i> group</b>
<i>G. austeni</i>	<i>G. caliginea</i>	<i>G. brevipalpis</i>
<i>G. longipalpis</i>	<i>G. fuscipes fuscipes</i>	<i>G. fuscipleuris</i>
<i>G. morsitans morsitans</i>	<i>G. fuscipes martini</i>	<i>G. fusca fusca</i>
<i>G. morsitans submorsitans</i>	<i>G. fuscipes quanzensis</i>	<i>G. fusca congolense</i>
<i>G. morsitans centralis</i>	<i>G. palpalis palpalis</i>	<i>G. haningtoni</i>
<i>G. pallidipes</i>	<i>G. palpalis gambiensis</i>	<i>G. longipennis</i>
<i>G. swynnertoni</i>	<i>G. pallicera pallicera</i>	<i>G. medicorum</i>
	<i>G. pallicera newsteadi</i>	<i>G. nashi</i>
	<i>G. tachinoides</i>	<i>G. nigrofusca nigrofusca</i>
		<i>G. nigrofusca hopkinsi</i>
		<i>G. severini</i>
		<i>G. schwetzi</i>
		<i>G. tabaniformis</i>
		<i>G. vanhoofi</i>
		<i>G. frezili</i>

**Table 1.1:**The 31 species of the genus *Glossina*

Tsetse flies are restricted to sub-Saharan Africa between latitudes 5°N and 20°S encompassing an area of 8.5 million km<sup>2</sup>, representing more than 40% of the total land area of 37 countries (Allsopp, 2001). The distribution of tsetse flies across sub-Saharan Africa and sleeping sickness foci are shown in Figure 1.3.





**Figure 1.3: Distribution of tsetse flies (in orange) and sleeping sickness foci (in blue) across sub-Saharan Africa (Kindly provided by Dr Eric Fèvre)**

#### **1.1.5.1 Tsetse biology**

Tsetse flies are obligate blood feeders; unlike mosquitoes, both male and female flies need to obtain blood meals every 4-5 days. Tsetse flies are unusual in their viviparous reproductive nature; a mated female produces a single egg every 9-10 d which, as it passes from the ovary to the uterus is fertilised by sperm stored in the spermatheca. The egg undergoes three moults within the female producing a third instar larva which is deposited. During development the larva is nourished by milk gland secretions from the mother. After deposition the larva burrows into soil where it pupates; tanning of the



larval cuticle, under the control of the hormone ecdysone occurs. Dependent on temperature an adult fly will emerge 30-36 days later, with female flies emerging several days before males. Laboratory grown females can produce more than 10 pupae, however, in the wild the number produced will be around three during the five week lifespan of the female tsetse. Male tsetse show shorter lifespans and generally live for around three weeks in the wild (Welburn and Maudlin, 1999).

#### 1.1.5.2 Organisms found within tsetse flies

Tsetse flies are host to a variety of different organisms (Figure 1.4). Three of these organisms are bacteria. *Wigglesworthia glossinidia* (Aksoy, 1995a) and *S. glossinidius* (Dale and Maudlin, 1999) belong to the  $\gamma$  proteo-bacteria with *Escherichia coli* the closest free-living relative, while *Wolbachia* (Cheng et al., 2000) is a member of the  $\alpha$  group and is closely related to the Rickettsia.

*W. glossinidia* the primary symbiont is located within specialised cells called mycetocytes, collectively called the mycetome, which is found surrounding the gut tissue located at the anterior end of the midgut (Aksoy, 1995a). The genome of *W. glossinidia* has recently been published (Akman *et al.*, 2002) and compared to *E. coli*, its closest living relative, *W. glossinidia* has a vastly reduced genome; however, the genes for production of B vitamins, essential for tsetse host nutrition and fecundity have been retained. The tsetse diet of whole blood is deficient in B vitamins and addition of antibiotics to the tsetse bloodmeal kills *W. glossinidia* resulting in sterility and reduced lifespans of asymbiotic progeny (Nogge, 1976). The addition of B vitamins to the bloodmeal of asymbiotic tsetse partially restores the ability to reproduce, suggesting that B vitamins are essential to the tsetse and are synthesised by *W. glossinidia* (Nogge, 1981).

*S. glossinidius* is found in all genera of tsetse residing both intra- and extra-cellularly in many different tissues of the fly including the midgut, milk glands and haemolymph (Cheng and Aksoy, 1999; Dale and Maudlin, 1999). No specific function has been

described for *S. glossinidius*, although it is thought to be involved in enhancing the flies' susceptibility to infection with trypanosomes (Maudlin and Dukes, 1985; Welburn *et al.*, 1993).

The *Wolbachia* of tsetse are closely related to the *Wolbachia* found in a wide variety of insects (Cheng *et al.*, 2000; Stouthamer *et al.*, 1999). The *Wolbachia* bacterium is associated solely with the reproductive tissues in both *G. m. morsitans* and *G. brevipalpis*; however, *Wolbachia* found in *G. austeni* are also found in somatic tissues (Cheng *et al.*, 2000). *Wolbachia* are associated with reproductive anomalies including cytoplasmic incompatibility (CI) and it has been estimated that they affect between 20-76% of insect species (Stouthamer *et al.*, 1999 Jeyaparakash and Hoy, 2000).

The remaining three organisms are usually only found in wild flies. The first and most important are members of the trypanosome family, which have been described above. The second is a parasitic nematode, *Hexameris glossinae* which initially invades larvae and is later found in the haemolymph (Moloo, 1972; Odindo and Hominick, 1985). Infections are associated with the rainy season, however; prevalence is quite low. In a recent study at Daloa in the Ivory Coast, 1.75% of *G. p. palpalis* examined were found to be infected (Dagnogo and Gouteux, 2003). The third organism found in tsetse is a baculovirus, which causes enlargement of the tsetse salivary glands, testicular degeneration and ovarian anomalies (Sang *et al.*, 1999).

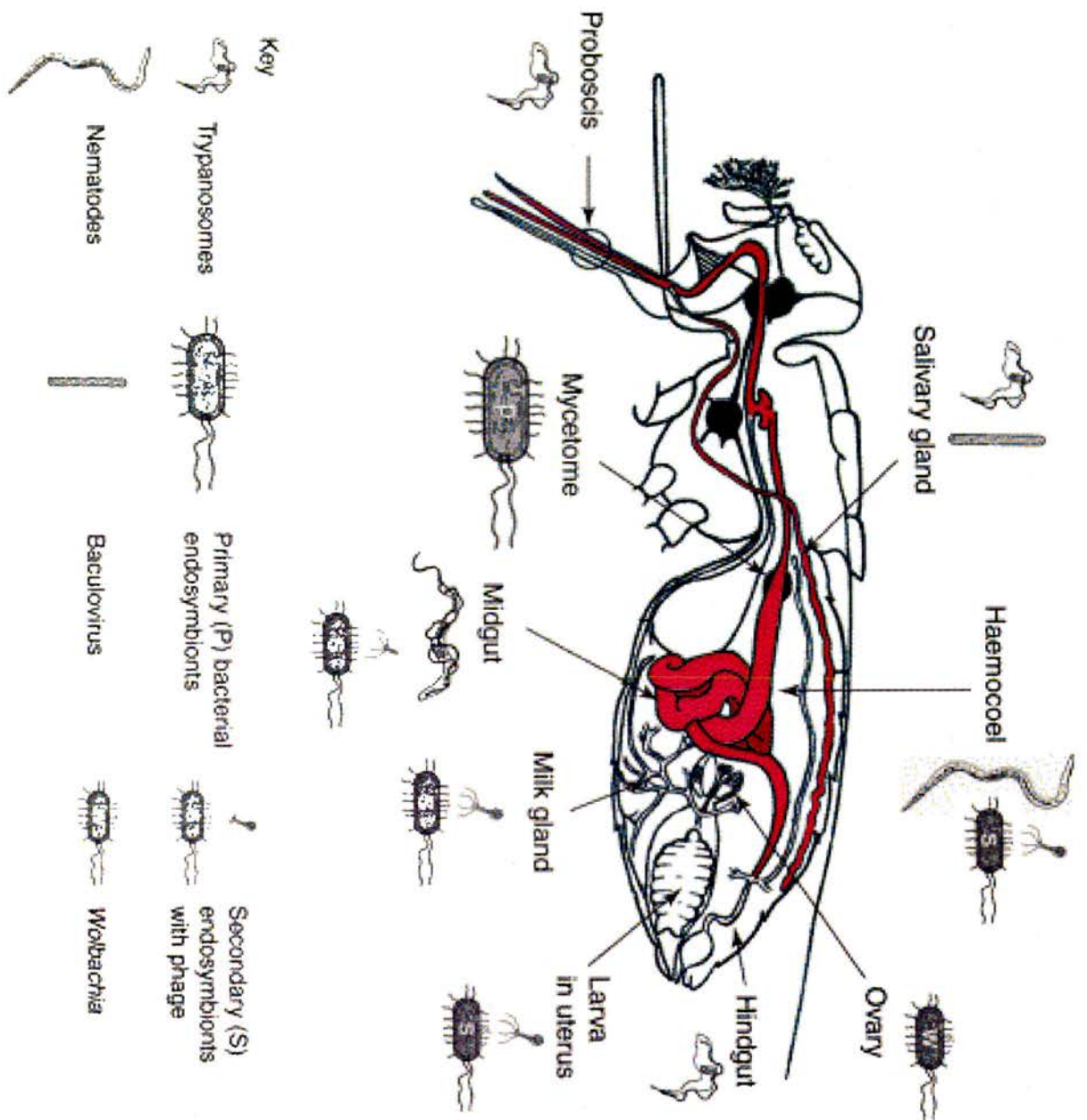


Figure 1.4: Organisms found within the tsetse fly (kindly provided by Dr Sue Welburn)

### 1.1.5.3 Tsetse bloodmeals

Tsetse flies can ingest a bloodmeal up to three times their normal weight, at which time they are at risk from predation. To reduce the weight of the bloodmeal, within 3 h of ingestion the water content of the bloodmeal is reduced from 79% to 55% (Bursell, 1960). Water moves from the bloodmeal to the haemolymph where the malphigian tubules, acting like mammalian kidneys, pump sodium ions into their lumen with water following by osmosis. Water then passes into the rectum and is excreted.

Although tsetse will feed on any available host, analyses of tsetse bloodmeals have shown that *G. austeni*, *G. fuscipleuris* and *G. longipennis* mainly feed on bushpig (*Potamochoerus porcus*) and *G. morsitans* spp. preferentially feed on warthogs (*Phacochoerus aethiopicus*). *G. longipalpis* and *G. fusca* preferentially feed on bushbuck (*Tragelaphus scriptus*) and *G. brevipalpis* feed mainly on hippopotamus (*Hippopotamus amphibious*; Clausen *et al.*, 1998). A study by Njiokou *et al.* (2004) found that *G. p. palpalis* fed mainly on humans (*Homo sapiens*) and antelopes. *G. f. fuscipes* has been shown to feed on monitor lizards (*Varanus exanthematicus*), with up to 27% of blood meals originating from lizards in some regions of Uganda (Waiswa *et al.*, 2003).

Once a suitable host has been found, the tsetse starts to probe the host's skin with its proboscis. Like most blood feeding insects, tsetse saliva has been shown to have vasodilatory and anticoagulant properties. The anticoagulant properties of tsetse saliva were first shown by Yorke and Macfie (1924) and it was later identified by Lester and Lloyd (1928) as a thrombin inhibitor. Two platelet aggregation factors have been found in *G. m. morsitans* saliva which inhibit thrombin induced aggregation (Mant and Parker, 1981). Saliva produced by sand flies has shown to have immunosuppressive and anti-inflammatory properties preventing adverse reactions in the host, and it is likely that tsetse saliva would also possess these characteristics (Kamhawi, 2000). Once blood is taken up it passes into the midgut; excess blood is stored in the crop, which is usually emptied 2 -3 h after feeding. Ingestion of the bloodmeal stimulates the production of

digestive enzymes and blood is digested in the posterior midgut; the production of enzymes is linked to the protein content of the bloodmeal (Gooding, 1974). Carboxypeptidases, proteinases, an aminopeptidase and haemolysin degrade the bloodmeal into free amino acids which are absorbed by the fly (Gooding, 1977a, b).

### **1.1.6 Tsetse control**

In the past many different control measures have been used to try and reduce the burden of trypanosomiasis in Africa. These include removing the tsetse vector by bush clearing, game destruction and the use of insecticides through aerial spraying. Currently though, most work undertaken to reduce trypanosome transmission is aimed at reducing the number of tsetse flies in an area using both odour-baited traps and insecticidal pour-ons; traps contain odours that attract tsetse and insecticides that kill them, while the latter uses insecticide treated cattle themselves as targets (Schofield and Maudlin, 2001).

#### **1.1.6.1 Bush clearing and game destruction**

Although these methods have been used successfully in the past to control tsetse (Swynnerton, 1921), the ecological implications of these approaches have ensured that bush clearing and game destruction are no longer used as frontline measures to reduce tsetse populations.

#### **1.1.6.2 Aerial spraying of insecticides**

Insecticides such as dichlorodiphenyl trichloroethylene (DDT) were used extensively to control tsetse populations during the 1950's and 1960's (Wilson, 1953; Glover *et al.*, 1960). However, concerns on the effects of organochlorine insecticides on the local wildlife led to them being withdrawn from use in developed countries in the 1970's,

however, their use continued in the developing world and they were used for tsetse control up to the 1990's (Wikteliu and Edwards, 1997). More recently synthetic pyrethroids such as deltamethrin and  $\lambda$ -cyhalothrin have been used in aerial and ground spraying strategies (Okoth *et al.*, 1991). Although insecticide resistance has arisen in mosquitoes (Hemingway, 1992), so far resistance has not developed in tsetse and computer models have shown that due to their low reproductive potential, insecticide resistance is unlikely to occur (Maudlin *et al.*, 1981).

#### **1.1.6.3 Traps and Targets**

Harris (1932; 1938) was the first to carry out large scale experiments in the use of traps to control tsetse populations. The discovery of baits both natural and synthetic has greatly improved the efficiency of traps suggesting that they may be used in control programs. The use of odour-baited fly-traps combined with synthetic pyrethroids provide 'removal trapping' involving visual, auditory and olfactory attractants along with insecticides, which has proved popular with control agencies (Day and Sjogren, 1994).

#### **1.1.6.4 'Pour on' or dipping of cattle with insecticides**

The insecticide deltamethrin used as a 'pour-on' on cattle has proved to be successful in decreasing tsetse populations and trypanosomiasis. Use of 'pour-on' reduced trypanosome infections rates in cattle from 37.7% to 2.9% and reduced the amount of tsetse caught in traps from 6.3 to 0.1 flies per trap per day (Okiria *et al.*, 2002). 'Pour ons' have the advantage of using a natural target to attract the tsetse, in this case the cow; when the tsetse lands it is killed by the insecticide. Dipping of cattle in deltamethrin has also proved highly effective in tsetse control in Uganda (Okello-Onen *et al.*, 1994)



#### 1.1.6.5 Transgenic methods

Transgenics, which involves the insertion and expression of a gene from one organism in another, has been put forward as a way of decreasing the vectorial capacity of disease carrying insects. Transformation of *Drosophila* was accomplished in 1982 with a P transposable element being inserted into the genome (Rubin and Spradling, 1982). In 1998 the first transformation of a mosquito was reported with the expression of the Hermes element from the housefly, which paved the way for investigation of insertion of genes which could block malaria transmission (Jasinskiene *et al.*, 1998). Several candidate genes have been tested, these include *SM1* (a salivary gland and midgut binding peptide) and *PLA2* (bee venom phospholipase A2; Moreira, 2003). *Rhodococcus rhodnii* the principle endosymbiont of *Rhodnius prolixus* the chief vector of Chagas disease has been transformed to produce the antimicrobial peptide L-cecropin and introduction of the transformed bacteria into *R. prolixus* renders the insect refractory to infection in 70% of cases and reduces the load of infection by a factor of 100 compared with insects carrying untransformed *R. rhodnii* (Beard *et al.*, 1998).

Due to the viviparous nature of tsetse reproduction, microinjection of foreign DNA into the intrauterine larva would be highly invasive and most likely to result in abortion. However, tsetse flies possess three endosymbiotic bacteria which could be transformed to produce antitrypanosomal molecules. *S. glossinidius* has been proposed as an excellent candidate for transformation in tsetse because of the proximity of the bacteria to the trypanosomes in the tsetse gut (Aksoy *et al.*, 2001). Culture of *S. glossinidius* is possible both in cell free medium and in co-culture with mosquito cells, allowing easy manipulation of the bacterium (Welburn *et al.*, 1987; Dale and Maudlin, 1999). The plant lectin concanavalin A has been proposed as a possible effector molecule due to its ability to induce programmed cell death in procyclic trypanosomes (Welburn *et al.*, 1999; Aksoy *et al.*, 2001). *S. glossinidius* has been transformed with a plasmid encoding genes for antibiotic resistance from the bacterium *Pseudomonas aeruginosa* (Beard *et al.*, 1993) as well as more recently with a plasmid encoding green fluorescent protein (Chen *et al.*, 1999). The latter were microinjected into female tsetse and were shown to be

acquired by both the F<sub>1</sub> and F<sub>2</sub> generations, showing that the transformed plasmid was stably transmitted. Once transformed *S. glossinidius* have been engineered, the next goal would be to replace the wild susceptible flies with their refractory counterparts. The use of the phenomenon of CI associated with the bacterium *Wolbachia* has been proposed as a possible method to drive the refractory phenotype through wild tsetse populations (Aksoy *et al.*, 2001). To achieve this, *Wolbachia* incompatible with wild type *Wolbachia* present in the natural tsetse population would have to be isolated. This could be accomplished by transferring a *Wolbachia* strain from another insect species, to which the tsetse had not been previously exposed or by combining two different *Wolbachia* infections to drive the maternal refractory genes through the population. Studies have shown that it is possible to transfer *Wolbachia* from one species to another with the *Wolbachia* phenotype being transferred as well (Braig *et al.*, 1994; McGraw *et al.*, 2002). However, care would have to be taken to stop susceptible flies acquiring these novel *Wolbachia* infections thereby allowing females to mate with transformed males, producing susceptible flies.

#### **1.1.6.6 Sterile insect technique**

The sterile insect technique (SIT) involves the release of high numbers of irradiated sterile males and has been used to successfully eradicate screwworm from North and Central America (Galvin and Wyss, 1994). As most female insects mate once then produce eggs from stored sperm, mating with a sterile male will result in the production of non-viable offspring. Although it has been shown that irradiated male tsetse were as efficient vectors of trypanosomes as non-irradiated males (Moloo *et al.*, 1988) a SIT program went ahead on the island of Zanzibar. The project started in 1994 involved the release of around 8.5 million sterilised male *G. austeni* and resulted in complete eradication by 1996 (Vreyson *et al.*, 2000). The program had the advantage of taking place on an island where only one species of tsetse was present and reinvasion of tsetse was unlikely. It has been estimated that it would cost US\$6.67 × 10<sup>10</sup> to use SIT against



a single tsetse species and where multiple species were involved it could take 350, 000 years to completely remove the flies from Africa (Molyneux, 2001).

#### 1.1.6.7 Treatment of trypanosomiasis

At present there are very few treatments available for sleeping sickness. Drugs that were introduced in the 1920's are still used today and only one drug has been registered for treatment against sleeping sickness in the last 50 years. Currently there are four drugs licensed for treatment of sleeping sickness (Barrett and Barrett, 2000): suramin (Fairlamb and Bowman, 1980), pentamidine (Sands *et al.*, 1985), melarsoprol (Friedheim, 1949) and D, L- $\alpha$ -difluoromethylornithine (DFMO; Bacchi *et al.*, 1980).

The process of antigenic variation has thwarted research into possible vaccines, with the continual changing of VSG making it unlikely that a vaccine derived from the major surface coat being of any use. Recent work has shown, however, that trypanosome microtubule-associated protein is a possible vaccine candidate, providing 100% protection to mice from a lethal dose of trypanosomes (Rasooly and Balaban, 2004). Suramin and pentamidine are used to treat the early stages of *T. b. rhodesiense* and *T. b. gambiense* infection respectively; however, these drugs do not cross the blood/brain barrier and therefore are not effective against the late stages of infection (Keiser *et al.*, 2001). Treatment of late stages of infection relies on melarsoprol, an arsenical-based drug, however, the drug is toxic and can cause death in up to 10% of patients (Keiser *et al.*, 2001). DFMO is used to treat melarsoprol refractory *T. b. gambiense* cases, however, it is expensive and requires a long course of treatment and shows little action against *T. b. rhodesiense* parasites (Burri and Brun, 2003).

Three compounds, isometamidium chloride, homidium and diminazene aceturate are used to treat animal trypanosomiasis (Leach and Roberts, 1981). Of these only isometamidium chloride has any useful long-term prophylactic effect providing at least 6 months of protection from *T. congolense* infection (Geerts *et al.*, 1999).

### 1.1.7 The current work

Due to the low trypanosome infection rates usually found in tsetse very little work has been done on why tsetse flies are such poor vectors of trypanosomes. In the 1920s it was shown that six out of seven flies infected with *T. brucei* were also infected with *T. congolense* and it was suggested that a certain number of individual flies were abnormally easy to infect (Buxton, 1955a). Later it was shown that susceptibility to trypanosome infection was a maternally inherited characteristic (Maudlin, 1982) and later the bacterial symbiont *S. glossinidius* was implicated as the effector organism (Welburn *et al.*, 1993). Experiments showed that the addition of the sugar glucosamine to the infective bloodmeal could inhibit the action of midgut lectins and artificially increase trypanosome infection rates (Maudlin and Welburn, 1987). It was then shown that *S. glossinidius* possessed chitinases capable of degrading chitin to glucosamine (Welburn *et al.*, 1993). At around the same time EM photographs of tsetse guts showed trypanosomes dying in a process similar to apoptosis (Welburn *et al.*, 1989) This led to the classical model which is based on the bacterial symbiont *S. glossinidius* producing inhibitory sugars which bind and inhibit trypanocidal lectins in the fly gut (Welburn *et al.*, loc. cit.). However, *S. glossinidius* is found within all laboratory colonies; with the same colonies producing low infection rates, possession of *S. glossinidius* alone is not the only factor involved in susceptibility to infection. Therefore to investigate the role played by *S. glossinidius* in susceptibility to trypanosome infection, the current work involves attempts to remove *S. glossinidius* from tsetse flies by the use of selective antibiotics.

Recently oxidative stress has been shown to play an important role in regulation of apoptosis. As commercially available lectins can induce programmed cell death in procyclic trypanosomes (Pearson *et al.*, 2000) and pro-oxidants are produced during bloodmeal breakdown (Souza *et al.*, 1997), the midgut environment is modulated by the addition of antioxidants to the bloodmeal. Cellular signalling by cyclic nucleotides is also important in the regulation of apoptosis and with cAMP signalling being important

in transformation of bloodstream form trypanosomes (Vassella et al., 1997), the effects of cAMP or cGMP on infection rates when added to the bloodmeal are also investigated. In the last 15 years there have been very few papers published on maturation of trypanosome infections in tsetse flies. It has been postulated that maturation is dependent on lectin activity (Welburn and Maudlin, 1989, 1990); the same sugars which increase the susceptibility of the tsetse to infection decrease the maturation rates of established midgut populations. This thesis investigates other factors which may affect maturation of midgut form trypanosomes. These include the effects of supplementing tsetse bloodmeals with antioxidants or cyclic nucleotides as well as investigating the effects of cold shock and mating on maturation rates.

### **1.18 List of objectives**

- 1 To create a line of *G. m. morsitans* negative for *S. glossinidius* with the use of the antibiotic streptozotocin.
- 2 Examine the effects of cyclic nucleotides on trypanosome infection rates by adding cAMP or cGMP to the infective bloodmeals of tsetse flies.
- 3 Examine the role of a variety of antioxidants on trypanosome infection rates by adding them to the infective bloodmeals of tsetse flies.
- 4 Examine dietary factors which may play a role in the trypanosome maturation process, by the addition of cyclic nucleotides or antioxidants to the bloodmeals.
- 5 Examine environmental and physiological factors which may play a role in the trypanosome maturation process.

## **Chapter 2**

### **Materials and methods**

## **2.1 Materials**

Materials were purchased from Watkins and Doncaster (PO Box 5, Cranbrook, Kent, TN18 5EZ, United Kingdom), Scientific Laboratory Supplies (SLS; Coatbridge Business Centre, 204 Main Street, Coatbridge, Lanarkshire, ML5 3RB, United Kingdom), VWR. (Merck House, Poole, Dorset, BH15 1TD, United Kingdom), Alpha Laboratories Limited, 40 Parham Drive, Eastleigh, Hampshire, SO50 4NU, United Kingdom), Whatman plc (27 Great West Road, Brentford, Middlesex, TW8 9BW, United Kingdom) Oxoid Ltd (Wade Road, Basingstoke, Hampshire, RG24 8PW, United Kingdom) and Camlab Ltd (Norman Way Industrial Estate, Over, Cambridge, CB4 5WE, United Kingdom).

## **2.2 Animals**

Calves were obtained from the Moredun Research Institute (Pentlands Science Park, Bush Loan, Penicuik, Midlothian, EH26 0PZ, United Kingdom). Sheep were obtained from Easter Bush Farm (Easter Bush, Roslin, EH25 9RG, United Kingdom). Mice were obtained from B and K Ltd (The Field Station, Grimston, Aldbrough, Hull, HU11 4QE, United Kingdom).

## **2.3 Chemicals**

Chemicals were purchased from Sigma Chemicals Ltd. (Fancy road, Poole, Dorset, BH12 4QH, United Kingdom), VWR. (Merck House, Poole, Dorset, BH15 1TD, United Kingdom), Gibco BRL Ltd. (3 Fountain Drive, Inchinnan Business Park, Paisley, PA4 9RF, United Kingdom), Bachem (Hauptstrasse 144, 4416 Bubendorf, Switzerland), Invitrogen (Invitrogen Ltd, 3 Fountain Drive, Inchinnan Business Park, Paisley, PA4

9RF United Kingdom) and Pharmacia and Upjohn Ltd. (Davy Avenue, Milton Keynes, MK5 8PH, United Kingdom).

## **2.4 Kits**

DNeasy Tissue Kit and Qiaquick Gel Extraction Kit were purchased from Qiagen (Qiagen house, Fleming Way, Crawley, West Sussex, RH10 9NQ, United Kingdom). The Wizard Plus Minipreps DNA purification system and pGEM<sup>®</sup>-T Easy Vector System were purchased from Promega UK Ltd (Delta House, Chilworth Science Park, Southampton, SO16 7NS, United Kingdom).

## **2.5 Oligonucleotides**

Oligonucleotides for use in polymerase chain reaction were synthesised by Sigma (Fancy road, Poole, Dorset, BH12 4QH, United Kingdom) and Promega. (Delta House, Chilworth Science Park, Southampton, SO16 7NS, United Kingdom).

## **2.6 Sterilisation**

Solutions and equipment unless otherwise stated were sterilised by autoclaving at 15 lb/in<sup>2</sup> at 121°C for 20 min. Trays used for tsetse feeding were sterilised by heating at 120°C for 4 h.

## **2.7 Media composition**

### **2.7.1 STE buffer**

100 mM NaCl (Sigma, UK), 10 mM Tris/HCL (Sigma, UK), 1 mM ethylenediaminetetraacetic acid (EDTA) at pH 8.0 (Sigma, UK).

### **2.7.2 LB medium**

10 g/l tryptone (Sigma, UK), 5 g/l yeast extract (Sigma, UK), 5 g/l NaCl (Sigma, UK).

### **2.7.3 LB plates with ampicillin/IPTG/X-Gal**

LB medium with the addition of 15 g/l agar (Sigma, UK) sterilised by autoclaving before addition of 100 µg/ml ampicillin (Sigma, UK), 0.5 mM Isopropyl β-D-1-thiogalactopyranoside (Sigma, UK) both filter sterilised and 80 µg/ml 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside (Sigma, UK)

### **2.7.4 SOC medium**

20 g/l tryptone (Sigma, UK), 5 g/l yeast extract (Sigma, UK), 10 ml/l 1 M NaCl (Sigma, UK), 2.5 ml/l 1 M KCl (Sigma, UK) sterilised by autoclaving before addition of 10 ml/l 2 M Mg<sup>2+</sup> (1 M MgCl<sub>2</sub>·6H<sub>2</sub>O, 1M MgSO<sub>4</sub>·7H<sub>2</sub>O; Sigma, UK) and 10 ml/l 2 M glucose (Sigma, UK) both filter sterilised.

### **2.7.5 Mitsuhashi and Maramorosch (MMI) medium**

Made to the recipe of Mitsuhashi and Maramorosch (1964).

8.125 g/l lactalbumin hydrolysate (Sigma, UK), 6.25 g/l yeastolate (Sigma, UK), 0.25 g/l  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  (Sigma, UK), 0.25 g/l KCl (Sigma, UK), 0.125 g/l  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  (Sigma, UK), 8.75 g/l NaCl (Sigma, UK), 0.15 g/l  $\text{NaHCO}_3$  (Sigma, UK) 0.28 g/l  $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$  (Sigma, UK) and 5 g/l D-glucose (Sigma, UK). Supplemented with heat-inactivated foetal calf serum (Invitrogen, UK) to 20% and filter sterilised.

### **2.7.6 Cunningham's medium**

Made to the recipe of Cunningham, (1977) by Gibco (UK).

0.53 g/l  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ , 3.04 g/l  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , 3.70 g/l  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 2.98 g/l KCl (anh.), 0.15 g/l  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.70 g/l Glucose, 0.40 g/l D-Fructose, 0.40 g/l Sucrose, 0.67 g/l L-malic acid, 0.37 g/l alpha-ketoglutaric, 0.055 g/l Fumaric acid, 0.06 g/l Succinic acid, 0.522 g/l Cis-aconitic acid, 10 ml/l Pyruvate (100 mM), 2.0 g/l  $\beta$ -Alanine, 1.09 g/l DL-alanine, 0.44 g/l L-arginine, 0.24 g/l L-asparagine.  $\text{H}_2\text{O}$ , 0.11 g/l L-aspartic acid, 0.08 g/l L-cysteine, 0.03 g/l L-cystine, 0.25 g/l L-glutamic acid, 1.64 g/l L-glutamine, 0.12 g/l glycine, 0.16 g/l L-histidine, 0.09 g/l DL-isoleucine, 0.09 g/l L-leucine, 0.15 g/l L-lysine, 0.20 g/l DL-methionine, 0.20 g/l L-phenylalanine, 6.90 g/l L-proline, 0.20 g/l DL-serine, 0.27 g/l L-aurine, 0.10 g/l DL-threonine, 0.10 g/l L-tryptophane, 0.20 g/l L-tyrosine, 0.21 g/l DL-valine, 2 ml/l BME Vitamins (100X), 4 ml/l Phenol red.

To make up Cunningham's medium 20% foetal calf serum (Sigma, UK) and 20  $\mu\text{g/ml}$  gentamicin (Invitrogen, UK) were added and the solution filter sterilised before use.



### **2.7.7 Phosphate Saline Glucose Glycerol (PSG G) 6:4**

8.088 g/l  $\text{Na}_2\text{HPO}_4$  (Sigma, UK), 0.359 g/l  $\text{NaH}_2\text{PO}_4$  (Sigma, UK), 2.5 g/l NaCl (Sigma, UK), 10 g/l Glucose (Sigma, UK) filter sterilised with the addition of 14% Glycerol (Sigma, UK).

## **2.8 Collection of blood**

### **2.8.1 Bovine blood**

Calves, three to four months old (Moredun Research Institute, UK) were rendered instantly insensible by captive bolt to the brain and pithed then bled by puncture of the jugular vein, blood was then passed into a defibrinating unit and allowed to defibrinate for 15 min before being separated into 100 ml aliquots and stored at 4°C for 5 d.

### **2.8.2 Ovine blood**

Ewes six to seven years old (Easter bush farm, UK) were rendered instantly insensible by captive bolt to the skull and bled by puncture of the jugular vein, blood was then passed into a defibrinating unit and allowed to defibrinate for 15 min before being separated into 100 ml aliquots and stored at 4°C for 5 d.

## **2.9 Tsetse fly origin and maintenance of flies**

### **2.9.1 Tsetse fly origin**

*Glossina morsitans morsitans* (Westwood) were originally from the Langford colony established at Bristol from pupae collected from Zimbabwe in 1967.

*Glossina morsitans centralis* (Machado) were kindly provided by the Livestock Research Institute (ILRI), Nairobi, Kenya.

*Glossina palpalis palpalis* (Robineau-Desvoidy) were kindly provided by the Seibersdorf Laboratories, International Atomic Energy Agency, Vienna, Austria.

### **2.9.2 Emergence of tsetse flies**

Tsetse flies were kept at  $25^{\circ}\text{C} \pm 1^{\circ}\text{C}$ , at 70% relative humidity. Pupae were collected from deposition trays and placed into an emergence cage. Once tsetse flies had emerged they were chilled at  $4^{\circ}\text{C}$  for 10 min and at this time were separated into males and females, placed in cages and allowed to rest for 24 h. Males were kept for 7-10 d before they are used for mating while females were mated immediately after their first bloodmeal. Flies were allowed to mate for 24 h unless otherwise stated then separated by chilling and fed the next day. Females were then placed on trays and pupae that were deposited were collected.

### **2.9.3 Feeding of tsetse flies**

Tsetse flies were fed through an artificial silicon membrane system (Mews, 1980), three to four times a week. Defibrinated blood was poured on to trays on heating blocks ( $37^{\circ}\text{C}$ ) and covered with a silicon membrane, caged tsetse flies were then placed onto the membrane covered with a dark cloth and allowed to feed for 10 min.

## **2.10 Isolation and cultivation of *S. glossinidius* from *G. m. morsitans* and *G. p. palpalis***

*S. glossinidius* was isolated from tsetse flies using the methods reported by Welburn *et al.* (1987) and Dale and Maudlin (1999). Newly emerged *G. m. morsitans* and *G. p.*

*palpalis* were surfaced sterilised in 5% sodium hypochlorite (VWR, UK) followed by rinsing in sterile saline. A leg of the tsetse was then pulled off and the haemolymph was collected using a sterile capillary tube (Camlab, UK). Immediately after collection, the haemolymph was mixed with 200 µl MMI medium containing 20% foetal calf serum (Invitrogen, UK). The mixture was then added to a flat bottomed centrifuge tube (VWR, UK) containing circular coverslips previously seeded with *Aedes albopictus* cell line C6/36 (European Collection of Cell Cultures (ECACC) Reference: 89051705) and centrifuged at 500 g for 10 min at 25°C to promote infection of insect cells. Cultures were then maintained at 25°C and passaged every 3 d by inoculation of a fresh C6/36 culture with 1/10 volume of *S. glossinidius* infected cells.

Insect cells containing *S. glossinidius* were plated on 10% packed horse blood MMI agar (1%) medium and cultivated under microaerophilic conditions generated using the Campygen pack system (Oxoid, UK) which provided 5% O<sub>2</sub>, balanced with CO<sub>2</sub> in sealed gas jars. A single colony was then isolated and transferred to MMI medium containing 20% foetal calf serum and grown in 25 cm<sup>2</sup> flasks (SLS, UK) at 25°C.

## **2.11 Sensitivity of *S. glossinidius* to antibiotics**

The antibiotic sensitivity of *S. glossinidius* was determined using the Kirby-Bauer disk diffusion method (Kirby *et al.*, 1966). Streptozotocin (Sigma, UK) and tetracycline (Sigma, UK) were made up in saline and filter sterilised. 10 µg or 25 µg of each antibiotic was then added to a filter disc (Whatman, UK) which was then placed on a 10% packed horse blood MMI agar (1%) plate inoculated with *S. glossinidius*. The growth of *S. glossinidius* was then assessed after 24 h at 25°C.

## **2.12 Addition of streptozotocin or tetracycline to bloodmeals**

### **2.12.1 Addition of streptozotocin 25 µg/ml or tetracycline 50 µg/ml**

Female teneral tsetse flies each were fed defibrinated ovine bloodmeals supplemented with streptozotocin 25 µg/ml (n=95; consisting of three replicates, comprising of one group of 35 flies and two groups of 30 flies) or tetracycline 50 µg/ml (n=95; replicates as for streptozotocin 25 µg/ml) and mated with male tsetse (7-10 d old) for 24 h then separated. Females then received three bloodmeals a week (Monday, Wednesday and Friday) supplemented with either streptozotocin at 25 µg/ml or tetracycline 50 µg/ml. Control flies (n=95; replicates as for streptozotocin 25 µg/ml) received unsupplemented bloodmeals and mated as above. Pupae that were deposited were collected and weighed then placed into labelled bijou tubes (SLS, UK) which had their tops perforated to allow air movement until emergence. Upon emergence they were sexed and frozen at -20°C for PCR analysis. Females that died were recorded.

### **2.12.2 Addition of streptozotocin 10 µg/ml or tetracycline 25 µg/ml**

Female teneral tsetse flies (n=90) were fed defibrinated ovine bloodmeals supplemented with streptozotocin 10 µg/ml (n=90; consisting of three replicates of thirty flies) or tetracycline 25 µg/ml (n=90; consisting of three replicates of thirty flies) and mated with male tsetse (7-10 d old) for 24 h then separated. Females then received three bloodmeals a week (Monday, Wednesday and Friday) supplemented with either streptozotocin at 10 µg/ml or tetracycline 25 µg/ml. Control flies (n=90; consisting of three replicates of thirty flies) received unsupplemented bloodmeals and mated as above. Pupae that were deposited were collected and weighed then placed into labelled bijou tubes (SLS, UK) which had their tops perforated to allow air movement until emergence. Upon

emergence they were sexed and frozen at -20°C for PCR analysis. Females that died were recorded.

### **2.12.3 Addition of streptozotocin 5 µg/ml or streptozotocin 10 µg/ml**

Female teneral tsetse flies were fed defibrinated bovine bloodmeals supplemented with streptozotocin 5 µg/ml (n=40) or streptozotocin 10 µg/ml (n=40) and mated with male tsetse (7-10 d old) for 24 h then separated. Females then received three bloodmeals a week (Monday, Wednesday and Friday) supplemented with either streptozotocin 5 µg/ml or streptozotocin 10 µg/ml. Control flies (n=40) received unsupplemented bloodmeals and were mated as above. After 12 d female tsetse were chilled and split into individual cages and fed as above until death. Pupae that were deposited were placed into bijou tubes (SLS, UK) which had their tops perforated to allow air movement. Upon emergence male flies were placed in individual cages and offered an unsupplemented bloodmeal three times a week (Monday, Wednesday and Friday) until death. Every second female fly was mated with a male tsetse (7-10 d old) and placed in individual cages and offered an unsupplemented bloodmeal three times a week (Monday, Wednesday and Friday) until death. Any pupae deposited were stored at -20°C for PCR analysis. Female flies that were not mated were placed in individual cages and offered an unsupplemented bloodmeal three times a week (Monday, Wednesday and Friday) until death.

### **2.13 Isolation of DNA from Tsetse Flies**

Two methods of DNA extraction were carried out: Phenol chloroform extraction and DNeasy Tissue Kit (Qiagen, UK).

### **2.13.1 Phenol chloroform extraction of DNA**

Frozen material was allowed to thaw and then homogenised in STE buffer with a polypropylene homogeniser then incubated in the presence of 0.5 µg/ml proteinase K (Sigma, UK) and 1% sodium dodecyl sulphate at 55°C for 24 h (Cheng and Aksoy, 1999). The tubes were centrifuged (7000 rpm) to pellet fly debris and the supernatant was removed. The corresponding value of phenol chloroform isoamyl (25:24:1; Sigma, UK) was then added to the supernatant and incubated at room temperature for 5 min. The samples were then spun at 3000 rpm for 3 min. If the top layer was clear it was transferred to a new tube and 1 volume of chloroform (Sigma, UK) was added. If the sample was cloudy the top layer was removed and 1 volume of STE buffer was added and phenol chloroform isoamyl was added as above and spun at 3000 rpm for 3 min. After addition of chloroform, samples were incubated for 3 min and were centrifuged as before. The top layer was removed and an equal volume of water saturated ether (Sigma, UK) was added to remove the chloroform. The samples were incubated and centrifuged as before, the top layer was removed and the samples were left open for 30 min to allow any remaining ether to evaporate. 0.5 volumes of 3 M sodium acetate (Sigma, UK) was added and the solution was mixed by gentle inversion. Four volumes of ice cold 100% ethanol (Sigma, UK) was added, mixed by inversion and stored over night at -20°C. The samples were then centrifuged at 3000 rpm for 10 min and the supernatant was removed. The samples were washed twice in 80% ethanol and then placed in a water bath at 50°C for 30 min to allow any remaining ethanol to evaporate. The samples were re-suspended in 100 µl TE (Sigma UK) and allowed to sit for 30 min before transfer into clean 1.5 ml Eppendorfs and storage at 4°C.

### **2.13.2 DNeasy Tissue Kit**

The instructions for Purification of Genomic DNA from Insects from the DNeasy tissue kit (Qiagen, UK) were followed, with homogenising of thawed tsetse in liquid nitrogen.

## 2.14 PCR analysis

### 2.14.1 Primers

Five primer sets were used in the current investigation (Table 2.1); these were 12SA1/12SB1 considered universal for insect mitochondrial DNA (O'Neill *et al.*, 1993), 170F/1227R targeted towards the 16S rDNA of *W. glossinidia* (Chen *et al.*, 1999), GP01F/GP01R designed to amplify a section of an abundant plasmid from *S. glossinidius*, wsp 81F/691R targeted to the *Wolbachia* Surface Protein (*wsp*) gene (Cheng *et al.*, 2000). During the current work primers were designed towards the chitinase gene of *S. glossinidius* (accession number Y11391).

Target DNA	Name	Sequence	Size	Reference
General insect primer	12SA1	5'-CTAGGATTAGATACCCTATT	400 bp	O'Neill <i>et al.</i> (1993)
	12SB1	5'-AAGAGCGACGGGCGATG		
<i>W. glossinidia</i>	170F	5'-ATAAAGCCTTGCGCTTTT	1.1 kbp	Chen <i>et al.</i> (1999)
	1227R	5'-CCATTGTAGCACGTGT		
<i>S. glossinidius</i>	GP01F	5'-TGAGAGGTTTCGTCAATGA	1.2 kbp	O'Neill <i>et al.</i> (1993)
	GP01R	5'-ACGCTGCGTGACCATTC		
<i>S. glossinidius</i>	C555F	5'-ACCGCCGCTTGTCGTT	555bp	
	C555R	5'-TCATAGGCGGTCGGGGATAAT		
<i>Wolbachia</i>	wsp 81F	5'-TGGTCCAATAAGTGATGAAGAAAC	610 bp	Cheng <i>et al.</i> (2000)
	wsp 691R	5'-AAAAATTAAACGCTACTCCA		

Table 2.1: Primers sets used for PCR analyses

### 2.14.2 Reaction conditions

The PCR reaction conditions were 5 min at 94°C followed by 35 cycles of 94°C, 55°C and 72°C at 1 min each followed by 5 min at 72°C. The PCR reaction mix contained 2.5

mM MgCl<sub>2</sub> (Sigma, UK), 0.25 mM dNTPs (Sigma, UK), primer concentrations of 500 nM and 1.5 units Redtaq (Sigma, UK). Reactions were carried out in either a Perkin Elmer 2400 or a MJ Research PTC 200 machine. A 10 µl sample was then loaded into a 1.5% TBE gel and run at 70-100V for 45-60 min and visualised after staining in ethidium bromide for 20 min followed by destaining in ddH<sub>2</sub>O for the same period.

## **2.15 Sequencing of PCR products**

### **2.15.1 Gel Extraction**

To extract PCR products from gels the Qiaquick Gel Extraction Kit protocol (Qiagen) was followed. Briefly, the DNA was excised from the gel using a clean scalpel and weighed. 3 volumes of Buffer QG was added to 1 volume of gel (100 µl Buffer QG 100 µg gel). The gel was then dissolved by incubating at 50°C for 10 min. 1 volume of isopropanol (Sigma, UK) was added and the sample was applied to a Qiaquick spin column and centrifuged for 1 min at 13000 rpm. 500 µl of Buffer QG was then added and centrifuged as before. The sample was then washed with Buffer PE and centrifuged. The spin column was then centrifuged to remove traces of Buffer PE and then added to a clean 1.5 Eppendorf tube and the DNA was eluted using buffer EB (10mM Tris-Cl, pH 8.5).

### **2.15.2 DNA ligation and transformation**

DNA ligations were carried out using pGEM-T Easy vector (Promega, UK) as per the manufacturer's instructions. Briefly, the amount of PCR product was determined using spectrophotometry and an amount corresponding to an insert:vector ratio of 3:1 was added and the mixture stored overnight at 4°C. The mixture was centrifuged and 2µl was added to 50 µl of *E. coli* (JM109) cells, stored on ice for 20 min and heat shocked at



42°C for 50 seconds. The mixture was then returned to ice for 2 min and incubated for 1.5 h in the presence of 950 µl of SOC medium at 37°C with shaking (150 rpm). 100 µl was then added and spread on LB/ampicillin/IPTG/X-Gal plates and incubated overnight at 37°C.

### **2.15.3 Isolation of plasmid DNA**

The Wizard Plus Minipreps DNA purification system (Promega) was used to isolate plasmid DNA. Briefly, a colony was picked from blue/white screening and transferred with a sterile pipette tip to 3 ml LB medium supplemented with ampicillin (100 µg/ml) and incubated overnight at 37°C with shaking (150 rpm). The cells were then pelleted by centrifugation at 2000 rpm for 10 min, resuspended in 300 µl cell resuspension solution (50mM Tris, pH 7.5; 10mM EDTA; 100µg/ml RNase A) and transferred to a sterile 1.5 ml micro-centrifuge tube. 300 µl of cell lysis solution (0.2M NaOH and 1% SDS) were added and inverted 4 times to mix the solution followed by addition of 300 µl neutralisation solution (1.32 M potassium acetate). The solution was then centrifuged for 5 min at 13000 rpm to pellet the cell debris. A wizard spin column was attached to a 2ml syringe (without the plunger) and 1 ml of resuspended resin was added. The supernatant was then transferred to the syringe barrel and the resin and pushed through the minicolumn using the syringe plunger. The mini column was then detached from the syringe barrel and the plunger removed, the minicolumn was then re-attached and 2 ml of column wash solution (80 mM Potassium acetate, 8.3 mM Tris-HCl, pH 7.5, 40 µM EDTA and 55% ethanol) was added and pushed through the spin column using the plunger. The spin column was then transferred to a microfuge tube and spun at 13000 rpm to dry the resin. The spin column was then transferred to a sterile microfuge tube and eluted with 50 µl H<sub>2</sub>O.

#### **2.15.4 Sequencing of plasmids**

Plasmids were sequenced by The Sequencing Service (Dundee, UK) and contigs were assembled using Lasergene (DNASTAR Inc) and screened against the nucleotide database at <http://www.ncbi.nlm.nih.gov/BLAST/>.

### **2.16 Trypanosome Production**

#### **2.16.1 Production of bloodstream form stabilates**

Two TO (BKI:BLW) mice (B and K Ltd, UK) were immunosuppressed by intraperitoneal injection of 80 mg/kg cyclophosphamide (Pharmica and Upjohn Ltd) and infected from a frozen trypanosome stabilate 24 h later by intraperitoneal injection. Regular checks were made by examination of blood by tail clipping of mice. When the parasitaemia reached between  $10^8$  and  $10^9$  /ml the blood from the two mice was collected by cardiac puncture under anaesthesia and pooled. The infected blood was then injected into 10 TO mice which had been immunosuppressed with cyclophosphamide (80 mg/kg) 24 h previously. Mice were then checked regularly and when parasitaemia reached between  $10^8$  and  $10^9$  /ml the blood from the ten mice was collected as above by cardiac puncture, pooled then diluted 1:1 in phosphate-buffered saline-glucose 6:4 (Lanham and Godfrey, 1970) with 14% glycerol. The mixture was then allowed to equilibrate for 10 min then separated into 200  $\mu$ l aliquots in 1 ml cryotubes (Nunc, Denmark) then frozen at  $-20^{\circ}\text{C}$  overnight in polystyrene boxes before transfer to  $-70^{\circ}\text{C}$ . This method yields a final dose of  $1 \times 10^6$  to  $4 \times 10^6$  /ml when the aliquot is reconstituted in 5 ml of blood (Welburn and Maudlin, 1987).

### **2.16.2 Culture of procyclic form trypanosomes**

Procyclic trypanosomes were obtained by infecting tsetse with bloodstream form trypanosomes and dissecting midguts from tsetse 10 d post-infection (Maudlin, 1996). Before dissection tsetse flies were sterilised with 5% sodium hypochlorite (VWR, UK) followed by rinsing in sterile saline. Trypanosome infected midguts were then placed in a 24 well plate (SLS, UK) in 1 ml Cunningham's medium (Gibco, UK) containing 20% foetal calf serum (Invitrogen, UK) and 120 µg/ml gentamicin (Sigma, UK). Once the procyclic culture was established it was transferred to 25 cm<sup>3</sup> culture flask (SLS, UK) and diluted every other day 1:10 in fresh Cunningham's medium containing 20% foetal calf serum and 20 µg/ml gentamicin.

## **2.17 Infection of tsetse flies with trypanosomes**

### **2.17.1 Trypanosome stocks used in the current work**

*T. b. brucei* isolate Butaba 135 (BUT 135) was isolated from a cow in Buteba village, Uganda in 1990 (Dale *et al.*, 1995)

*T. b. brucei* isolate Gutat 3.1 (GUT 3.1) was derived from TrEU 667 (Black *et al.*, 1982)

*T. b. rhodesiense* isolate Dyphasi Obwang (DO) was isolated from a human patient during a sleeping sickness outbreak in Southeast Uganda in 1990 (Welburn *et al.*, 1999)

### **2.17.2 Infection of tsetse flies from bloodstream stabulates or from procyclic culture**

Tsetse flies to be infected had their wings clipped on emergence and allowed to rest for 24 h (unless otherwise stated) before being infected with trypanosomes. To make up the

infective bloodmeal from a stabilate, it was defrosted at room temperature then reconstituted in 5 ml of ovine blood. For procyclic infections, cultured procyclics were centrifuged at 2500 rpm for 5 min and the culture supernatant removed, trypanosomes were then resuspended in 5 ml of ovine blood at a concentration of  $1 \times 10^6$  to  $4 \times 10^6$  /ml. Test compounds were made up in sterile saline at differing concentrations and were then added to the infective bloodmeal. The bloodmeal was then placed on a heated tray and covered with a silicon membrane. Tsetse were then placed on the membrane and allowed to feed. Flies that did not feed were removed from the experiment.

### **2.17.3 Dissection of tsetse flies**

Tsetse flies were chilled at 4°C for 30 min then kept on ice until dissection. Flies were dissected in saline either 10 d (midgut examination), 14 d, 21 d or 28-30 d (midgut and salivary gland examination) post-infection and examined for the presence of trypanosomes by phase-contrast microscopy (X400).

## **2.18 Midgut infections of bloodstream form *T. b. brucei* in *G. m. morsitans***

### **2.18.1 Teneral infections of *G. m. morsitans* with *T. b. brucei***

Teneral male *G. m. morsitans* received an infective bloodmeal containing *T. b. brucei* isolate BUT 135 supplemented with one of the following compounds: glutathione (GSH; Sigma, UK) at 1 mM, 5 mM or 10 mM; 15 mM glutathione disulphide (GSSG; Sigma, UK); L- or D-cysteine (Sigma, UK) at 1mM, 5 mM or 10 mM; N-acetyl-cysteine (NAC; Sigma, UK) at 0.1 mM, 0.5 mM or 1 mM; uric acid (Sigma, UK) at 1 mM, 10 mM or 20 mM; ascorbic acid (Sigma, UK) at 1 mM, 10 mM or 20 mM; 8-Bromo-cyclic guanosine monophosphate (8-Br-cGMP; Sigma, UK) at 1  $\mu$ M, 10  $\mu$ M or 100  $\mu$ M; 8-Bromo-cyclic

adenosine monophosphate (8-Br-cAMP; Sigma, UK) at 1  $\mu$ M, 10  $\mu$ M or 100  $\mu$ M; 1 mM cyclic guanosine monophosphate (cGMP; Sigma, UK); 10 mM L-cystine (Sigma, UK); 2 mg catalase (Sigma, UK); 2 mg superoxide dismutase (Sigma, UK); 8 mM trypanothione (Bachem, Switzerland); 20 mM glutamic acid (Sigma, UK); 1 mM serotonin (Sigma, UK); 15 mM ornithine (Sigma, UK); EDTA (Sigma, UK) at 1 mM or 10 mM; 1 mM nitrilotriacetic acid (Sigma, UK); 15 mM aminoguanidine (Sigma, UK). Control flies received unsupplemented infective feeds.

Teneral female *G. m. morsitans* received an infective bloodmeal containing *T. b. brucei* isolate BUT 135 supplemented with one of the following compounds; GSH (Sigma, UK) at 1 mM, 5 mM or 10 mM; 8-Br-cGMP (Sigma, UK) at 1  $\mu$ M, 10  $\mu$ M or 100  $\mu$ M; uric acid (Sigma, UK) at 1 mM, 10 mM or 20 mM. Control flies received unsupplemented infective feeds.

Teneral male and female *G. m. morsitans* received an infective bloodmeal containing *T. b. brucei* isolate GUT 3.1 supplemented with 1 mM cGMP. Control flies received unsupplemented infective feeds.

### **2.18.2 Non-teneral infections of *G. m. morsitans* with *T. b. brucei***

Teneral male *G. m. morsitans* were fed on uninfected ovine blood and were then infected 3 d later with bloodmeals containing *T. b. brucei* isolate BUT 135 supplemented with one of the following compounds; 8-Br-cGMP at 100 or 200  $\mu$ M; GSH at 10 or 15 mM; 20 mM ascorbic acid; 20 mM uric acid. Control flies received an unsupplemented infective feed.

### **2.18.3 Non-teneral *G. m. morsitans* infected with *T. b. brucei* when the teneral bloodmeal was supplemented with 8-Br-cGMP or GSH**

Teneral male *G. m. morsitans* were fed on uninfected ovine blood supplemented with either 100  $\mu$ M 8-Br-cGMP or 15 mM GSH, then infected at the second feed with an unsupplemented bloodmeal containing *T. b. brucei* isolate BUT 135. Control flies received an unsupplemented bloodmeal as teneral and an unsupplemented infective bloodmeal at the second feed containing *T. b. brucei* isolate BUT 135.

### **2.18.4 Addition of 8-Br-cGMP, GSH or NAC at the second feed to *G. m. morsitans* infected as teneral with *T. b. brucei***

Teneral male *G. m. morsitans* were infected with *T. b. brucei* isolate BUT 135 and then fed either 100  $\mu$ M 8-Br-cGMP, 15 mM GSH or 5 mM NAC at the second feed ranging from 48 h to 120 h post-infection. Control flies received an unsupplemented bloodmeal at the second feed. Tsetse flies were then dissected and midguts were examined 10 d after receiving their second bloodmeal.

### **2.18.5 Injection of 8-Br-cGMP, GSH or NAC into *G. m. morsitans* followed by infection with *T. b. brucei***

Teneral female *G. m. morsitans* were chilled for 5 min at 4°C then kept on ice until injected. Using a dose calculated on a on a bloodmeal size of 30  $\mu$ l, 8-Br-cGMP, GSH or NAC was dissolved in saline, filter sterilised and the equivalent of a bloodmeal dose of 200  $\mu$ M 8-Br-cGMP (3.3  $\mu$ g), 15 mM GSH (150  $\mu$ g) or 1 mM NAC (5.5  $\mu$ g) respectively was injected under the scutellum. Flies then received an unsupplemented infective bloodmeal 8 h later containing *T. b. brucei* isolate BUT 135.

#### **2.18.6 Injection of *E. coli* into *G. m. morsitans* followed by infection with *T. b. brucei***

A single colony of *E. coli* (obtained from the National Collection of Industrial and Marine Bacteria; strain 9481) was picked from a 10% packed horse blood MMI agar (1%) plate and grown in a LB medium overnight. The following day the bacterial culture was spun down (3000 rpm) and washed three times in saline and finally resuspended in saline at OD<sub>600</sub>=0.4. Teneral female *G. m. morsitans* were then injected with 2 µl *E. coli* suspension under the scutellum. 24 h later the flies were provided with an infective bloodmeal containing *T. b. brucei* isolate BUT 135 supplemented with either NAC (1 mM or 5 mM) or 8-Br-cGMP (50 µM). Control flies were injected with saline.

#### **2.18.7 Injection of *S. glossinidius* into *G. m. morsitans* followed by infection with *T. b. brucei***

Cultures of *S. glossinidius* derived from either *G. m. morsitans* and *G. p. palpalis* were grown in MMI medium for 3 d then spun down (3000 rpm) and washed three times in saline and finally resuspended in saline at OD<sub>600</sub>=0.4. Teneral female *G. m. morsitans* were then injected with 2 µl *S. glossinidius* suspension from either *G. m. morsitans* or *G. p. palpalis* under the scutellum. Flies then received an infective bloodmeal 24 h later containing *T. b. brucei* isolate BUT 135 supplemented with 0.8 mM NAC. Control flies were injected with saline.

#### **2.18.8 Addition of *E. coli* to the bloodmeal of *G. m. morsitans* followed by infection with *T. b. brucei***

Teneral male *G. m. morsitans* received a bloodmeal supplemented with  $10^6$  cells/ml *E. coli* (strain 9481) in saline then 3 d later they were allowed to feed on an infective bloodmeal containing *T. b. brucei* isolate BUT 135 supplemented with either NAC (1 mM or 5 mM) or 8-Br-cGMP (50  $\mu$ M or 100  $\mu$ M) or an unsupplemented diet of ovine blood. Control flies received the same amount of saline in the infective bloodmeal.

### **2.19 Midgut infections with procyclic form *T. b. brucei* in *G. m. morsitans***

#### **2.19.1 Teneral infections of *T. b. brucei* in *G. m. morsitans***

Teneral male *G. m. morsitans* received an infective bloodmeal containing procyclic *T. b. brucei* isolate BUT 135 supplemented with 15 mM GSH.

#### **2.19.2 Incubation of procyclic form *T. b. brucei* with 8-Br-cGMP followed by infection in *G. m. morsitans***

Procyclic form *T. b. brucei* isolate BUT 135 trypanosomes were incubated with 200  $\mu$ M 8-Br-cGMP for 1 h. Incubated trypanosomes were then centrifuged at 2500 rpm washed three times in unsupplemented Cunningham's medium and finally resuspended in 5 ml of ovine blood. Control procyclics and procyclics to which 200  $\mu$ M 8-Br-cGMP was added to the bloodmeal received the same washes as those which had been incubated in 8-Br-cGMP. Teneral male *G. m. morsitans* were then allowed to feed on the infective bloodmeals.



## **2.20 Midgut infections with bloodstream form *T. b. rhodesiense* in *G. m. morsitans***

Teneral male *G. m. morsitans* received an infective bloodmeal containing *T. b. rhodesiense* isolate DO supplemented with the following compounds: GSH at 15 mM; ascorbic acid at 15 mM; 8-Br-cGMP at 100  $\mu$ M.

Teneral female *G. m. morsitans* received an infective bloodmeal supplemented with the following compounds; 20 mM uric acid; 100 mM 8-Br-cGMP. Control flies received unsupplemented infective feeds.

## **2.21 Midgut infections with bloodstream form *T. b. brucei* in *G. m. centralis***

Teneral male and female *G. m. centralis* received an infective bloodmeal containing *T. b. brucei* isolate GUT 3.1 supplemented with 1 mM cGMP.

## **2.22 Midgut infections with bloodstream form *T. b. brucei* in *G. p. palpalis***

Teneral female *G. p. palpalis* received an infective bloodmeal containing *T. b. brucei* isolate BUT 135 supplemented with one of the following compounds: GSH at 5 mM, 10 mM or 15 mM; NAC at 1 mM or 5 mM; uric acid at 20 mM; ascorbic acid at 30 mM; 8-Br-cGMP at 200  $\mu$ M. Control flies received unsupplemented infective feeds.

## **2.23 Midgut infections with bloodstream form *T. b. rhodesiense* in *G. p. palpalis***

Teneral male *G. p. palpalis* received an infective bloodmeal containing *T. b. rhodesiense* isolate DO supplemented with 200  $\mu$ M 8-Br-cGMP. Control flies received unsupplemented infective feeds.

## **2.24 Maturation of *T. b. brucei* infections in *G. m. morsitans***

### **2.24.1 Single dose infections with 8-Br-cGMP or GSH on infection rates of *T. b. brucei* in *G. m. morsitans***

Male and female *G. m. morsitans* received an infective bloodmeal containing *T. b. brucei* isolate BUT 135 supplemented with either 100  $\mu$ M 8-Br-cGMP or 15 mM GSH. Control flies received an unsupplemented infective bloodmeal.

### **2.24.2 Infective bloodmeal containing 100 $\mu$ M 8-Br-cGMP followed by the addition of other compounds on the infection rates of *T. b. brucei* in *G. m. morsitans***

Male and female *G. m. morsitans* received infective bloodmeals containing *T. b. brucei* isolate BUT 135 supplemented with 100  $\mu$ M 8-Br-cGMP and were then subsequently fed on bloodmeals containing one of the following compounds: 100  $\mu$ M 8-Br-cAMP, 15 mM GSH, 10 mM L-cysteine, 10 mM D-cysteine, 5 mM cystine, 10 mM uric acid, 20 mM ascorbic acid, 5 mM L-N-nitro-arginine-methyl-ester (NAME), 5 mM D-NAME, 10 mM glutamic acid, 10 mM glycine or 20 mM proline.

Male *G. m. morsitans* received infective bloodmeals containing *T. b. brucei* isolate BUT 135 supplemented with 100  $\mu$ M 8-Br-cGMP and were then subsequently fed on one of the following compounds: 1 mM NAC, 15 mM L-arginine, 15 mM D-arginine, 15 mM aminoguanidine. Control flies received unsupplemented ovine blood after receiving an infective feed containing 100  $\mu$ M 8-Br-cGMP.

Male flies which had received infective bloodmeals supplemented with 100  $\mu$ M 8-Br-cGMP and then continually fed on bloodmeals containing 100  $\mu$ M 8-Br-cGMP were dissected after 14 d.

#### **2.24.3 Infective bloodmeal containing 15 mM GSH followed by the addition of other compounds on the infection rates of *T. b. brucei* in *G. m. morsitans***

Male and female tsetse flies received an infective bloodmeal containing *T. b. brucei* isolate BUT 135 supplemented with 15 mM GSH and were then subsequently fed on bloodmeals containing 100  $\mu$ M 8-Br-cGMP or 100  $\mu$ M 8-Br-cAMP. Control flies received unsupplemented ovine blood after being provided with an infective bloodmeal containing 15 mM GSH.

Male flies which had received infective bloodmeals supplemented with 15 mM GSH and then subsequently fed on bloodmeals containing 100  $\mu$ M 8-Br-cGMP or 100  $\mu$ M 8-Br-cAMP were dissected after 14 d.

#### **2.24.4 Effects of temperature on maturation of *T. b. brucei* in *G. m. morsitans***

Male and female *G. m. morsitans* received an infective bloodmeal containing *T. b. brucei* isolate BUT 135 supplemented with 15 mM GSH. After 72 h flies received a cold shock at 4°C for 30 min and were then returned to 25°C. Flies were allowed 24 h rest then allowed to feed on unsupplemented ovine blood. Control flies did not receive a cold shock.

To examine if chilling for different lengths of time affected maturation rates of trypanosome infections, flies were chilled at 4°C for 5 min, 1 h or 3 h and examined for trypanosomes 21 d post-infection.

#### **2.24.5 Effects of mating on maturation of *T. b. brucei* in female *G. m. morsitans***

Female *G. m. morsitans* received an infective bloodmeal containing *T. b. brucei* isolate BUT 135 supplemented with 15 mM GSH and were then mated with 7-10 d old males for 72 h. Flies were then chilled at 4°C for 30 min to allow separation of males and females. Control females were unmated but were chilled in the same way as mated females.

### **2.25 Maturation of *T. b. rhodesiense* infections in *G. m. morsitans***

#### **2.25.1 Infective bloodmeal containing 100 µM 8-Br-cGMP**

Male and female *G. m. morsitans* received an infective bloodmeal containing *T. b. rhodesiense* isolate DO supplemented with 100 µM 8-Br-cGMP and were then fed 10 mM L-cysteine.

#### **2.25.2 The effects of temperature on maturation of *T. b. rhodesiense* in *G. m. morsitans***

Male and female *G. m. morsitans* received an infective bloodmeal containing *T. b. rhodesiense* isolate DO supplemented with 15 mM GSH. After 72 h flies received a cold shock at 4°C for 30 min and were then returned to 25°C. Flies were allowed 24 h rest

then allowed to feed on unsupplemented ovine blood. Control flies did not receive a cold shock.

## 2.26 Statistical analyses

### 2.26.1 Number of pupae produced

To investigate if streptozotocin or tetracycline had any effect on the number of pupae produced, the data from the antibiotic groups were compared to the control data using generalised linear models with Poisson errors taking into account potential replicate differences in R version 1.9.1 (R project). Generalised linear models with Poisson errors allow the examination of datasets containing integers (Crawley, 2002) as tsetse cannot deposit a fraction of a pupa. Analysis was carried to determine if antibiotic treatment significantly affected the total number of pupae deposited during the entire experiment, the number of pupae deposited during the first larval cycle and the number of pupae deposited during the second larval cycle. The code used for analysis is shown in Box 2-1 and differences were considered significant at  $p < 0.05$ .

```
anova(glm(pupae~rep+treatment+rep:treatment,data=numberofpupae,family="poisson"),test="Chisq")
```

**Box 2-1:** Code used for analysis of the number of pupae deposited (**glm**: generalized linear model; **pupae**: number of pupae deposited; **rep**: replicate number; **treatment**: control, streptozotocin or tetracycline groups; **data**: the dataset analysed).

### 2.26.2 Time to deposit the first pupae

To investigate if streptozotocin or tetracycline had an effect on the time taken to deposit the first pupae (calculated by subtracting the date of mating from the date of pupal

deposition), the data from the antibiotic groups were compared against the control data using generalised linear models with poisson errors taking into account potential replicate differences in R. As above, generalised linear models with poisson errors were used to examine the dataset (as you cannot have less than a whole day). Due to significant differences being found between low pupal weight and unsuccessful emergence (see section 3.2.6) only pupae that emerged successfully were used for analysis. The code used for analysis is shown in Box 2-2 and differences were considered significant at  $p < 0.05$ .

```
anova(glm(deposition~rep+treatment+rep:treatment,family="poisson",data=depositiontime),test="Chisq")
```

**Box 2-2: Code used for analysis of the time taken to deposit the first pupae (glm: generalized linear model; deposition: time taken to deposit the first pupae; rep: replicate number; treatment: control, streptozotocin or tetracycline groups; data: the dataset analysed).**

### 2.26.3 Pupal weights

Prior to analysis being carried on treatment and sex effects it was found that there were significant differences in the pupal weights of flies which emerged and those which failed to emerge (see chapter 3.2.6). Therefore the analysis of pupal weights investigating the effect of antibiotic treatments or the sex of the pupae were only carried out on those pupae that emerged successfully. To investigate if streptozotocin or tetracycline had an effect on pupal weight, the data from the antibiotic groups were compared with the control data taking into account replicate differences using analysis of variance (ANOVA) in R. Due to the fact that antibiotic treatment virtually stopped pupal production after the first larval cycle (see chapter 3.2.2), only pupae obtained from the first larval cycle were used for analysis. To examine if the sex of the pupae had an effect on its deposited weight all pupae obtained that emerged were subjected to analysis

by ANOVA in R. The codes used for analysis is shown in Box 2-3 and differences were considered significant at  $p<0.05$ .

```
summary(aov(weight~rep+treatment+rep:treatment,data=pupalweight))
summary(aov(weight~sex+treatment+rep:treatment+treatment:sex+sex:treatment:rep+sex:rep,data=sexpupalweight))
```

**Box 2-3:** Codes used for analyses of pupal weights (aov: analysis of variance; weight: weight of deposited pupae; rep: replicate number; treatment: control, streptozotocin or tetracycline groups; sex: sex of the pupae (determined after emergence); data: the dataset analysed).

#### 2.26.4 Time to emergence from the puparium

To investigate if streptozotocin or tetracycline had an effect on the time taken for the pupae from the first larval cycle to emerge (the date the pupae was deposited subtracted from the date of emergence), the data from the antibiotic groups of each sex was compared against the control data in R using generalised linear models with poisson errors taking into account potential replicate differences. As with the time to deposit the first pupae, generalised linear models with poisson errors were used to examine the dataset (as you cannot have less than a whole day). The data for female and male flies was analysed separately as previous studies had shown significant differences in the time taken for female and male flies to emerge (Buxton, 1955b; Birkenmeyer and Dame, 1975). The code used for analysis is shown in Box 2-4 and differences were considered significant at  $p<0.05$ .

```
anova(glm(deposition~rep+treatment+rep:treatment,family="poisson",data=depositiontime),test="Chisq")
```

**Box 2-4:** Code used for analysis of emergence time from the pupae (glm: generalised linear model; emergence: time taken from deposition to emergence; rep: replicate; treatment: control, streptozotocin or tetracycline group; data: the dataset analysed).

### 2.26.5 Percentage of flies emerging successfully from the puparium

To investigate the affect of streptozotocin or tetracycline on the percentage of pupae to emerge, the data from the antibiotic groups was compared to the control data using logistic regression in R. Analysis was carried out to determine if antibiotic treatment significantly affected the number of flies to successfully emerge from pupae deposited during the first larval cycle as well as the number of flies that emerged from all pupae deposited during the entire experiment. The code used for analysis is shown in Box 2-5 and differences were considered significant at  $p<0.05$ .

```
anova(glm(cbind(emrpos,emrneg)~treatment+rep+treatment:rep,data=emrposneg,family="binomial"),test="Chisq")
```

**Box 2-5:** Code used for the analysis of the emergence of pupae between treatments (glm generalised linear model; cbind: R function to make columns; emrpos: number of pupae successfully emerging; emrneg: number of pupae failing to emerge; treatment: control, streptozotocin or tetracycline groups; rep: replicate number; data: the dataset analysed).



### 2.26.6 Survival analyses

To investigate if streptozotocin or tetracycline affected the lifespan of tsetse, the data from the antibiotic groups were compared with control data using the Kaplan-Meier survival estimate in R (Parmar and Machin, 1995). Survival analyses can take into account subjects which had not died during the study, however, during the current work all flies died. The code used for analysis is shown in Box 2-6 and differences were considered significant at  $p < 0.05$ .

```
survdif(Surv(days,death)~treatment,data=lifespan)
```

**Box 2-6:** Code used for analysis of survival times (Surv: R survival curve fitting function; survdiff: function to test differences between fitted survival curves; days: lifespan of the fly; death: whether the fly was alive or dead at the end of the experiment – in this case they were all dead; treatment: control, streptozotocin or tetracycline groups; data: the dataset analysed)

### 2.26.7 Trypanosome infection rates

To investigate if the test compounds (described in chapter 2.19 and 2.20) affected midgut infection rates or transmission of infectivity (TI, calculated as the proportion of salivary gland infections maturing from established midgut infections (Welburn *et al.*, 1995)),  $\chi^2$  tests conducted in Minitab version 14 (Minitab, Inc.) were used to compare the number of control flies with midgut infections against the number of treated flies with midgut infections as well as the proportion of midgut infections that matured in the control groups against the proportion of midgut infections that matured in the treated groups. Differences were considered significant at  $p < 0.05$ .

### 2.26.8 Re-examination of Otieno *et al.* (1983)

Otieno *et al.* (1983) infected male *G. m. morsitans* with *T. b. brucei* stock EATRO 1969 and investigated the effect of chilling (in two age groups 1-8 h and 12-24 h old) 30 min after bloodmeal ingestion on the subsequent midgut and salivary gland infection rates (see Appendix 1 for details). However, Otieno *et al.* (loc. cit) when comparing salivary gland infection rates used the total number of flies dissected as denominator rather than the number of midgut infections which had matured into salivary gland infections. During the current work it was felt that the data from Otieno *et al.* (loc. cit.) needed to be re-analysed, taking into account the number of midgut infections progressing to salivary gland infections rather than the number of salivary gland infections from the total number of flies dissected. In order to take into account the two age groups when comparing the effect of chilling on both midgut infection rates and TI, logistic regression was undertaken in R as outlined in Box 2-7 and differences were considered significant at  $p < 0.05$ .

```
(a) summary(glm(cbind(mg,n-mg)~agegroup*treatment,family="binomial",data=otieno1983))
(b) summary(glm(cbind(sg,mg-sg)~agegroup*treatment,family="binomial",data=otieno1983))
```

**Box 2-7: Codes used for analyses of Otieno *et al.* (1983) data (a) midgut infections (b) transmission of infectivity (glm: generalized linear model; cbind: R function to make columns; mg: number of midgut infections; sg: number of salivary gland infections; n: number of flies dissected; agegroup: age of flies when infected; treatment: control or chilled groups; data: the dataset analysed).**

## **Chapter 3**

### **Effects of antibiotics on symbionts and fitness parameters of *G. m. morsitans***

### 3.1 Introduction

Although bacteria are found within many insects, only around 10% of insects are thought to live in a symbiotic relationship with bacteria (Douglas, 1989). Insects which rely on symbionts generally live on diets that are unbalanced or nutritionally poor and are usually found in four orders: the Dictyoptera (cockroaches), Hemiptera (psyllids and aphids), Phthiraptera (lice) and Coleoptera (beetles), although some members of the Diptera and Hymenoptera also contain symbionts. Symbiotic bacteria are usually intracellular and live in specialised cells called mycetocytes. Buchner (1965) speculated that because insects with symbionts thrive on comparatively poor diets their bacterial symbionts must be producing nutrients which are lacking in the insects' diet. Aphid and tsetse diets of plant sap and vertebrate blood are deficient in nitrogenous amino acids and B vitamins respectively whilst the termite diet of wood is nutritionally poor. The addition of antibiotics to the diet of pregnant aphids and tsetse flies results in the production of short-lived sterile offspring, suggesting that the symbionts are essential for host viability (Hill *et al.*, 1973; Griffiths and Beck, 1974; Nogge, 1976). Bacteria that are essential to the viability of the insect are defined as primary bacteria, while bacteria that are not essential and are not found in every individual of the population are called secondary bacteria (Douglas, 1989). Bacteria may also be harmful to the insect, in which case they are called parasitic (Stouthamer *et al.*, 1999).

It is thought that symbiosis between insects and bacteria evolved when bacteria invaded ancestors of the insects involved. Current phylogenetic evidence suggests that the primary symbionts of tsetse (Chen *et al.*, 1999), aphids (Funk *et al.*, 2000) and carpenter ants (Sauer *et al.*, 2000) show concordance with their insect hosts, suggesting that the primary symbiont and the insect have evolved together. The aphid/*Buchnera* symbiosis, both by analysis of bacterial sequences and fossil evidence is thought to be 200-250 million years old, beginning around the time of the origin of the Aphidoidea (Funk *et al.*, 2000).

### 3.1.1 The bacterial symbionts of tsetse flies

#### 3.1.1.1 *Wigglesworthia glossinidia*

The primary symbiont of the tsetse fly, *Wigglesworthia glossinidia*, is a gram-negative, rod shaped bacterium, 8-10 µm long, 1.5-2 µm wide and appears to divide by binary fission (Aksoy, 1995a). *W. glossinidia* is found within mycetocytes which form the mycetome, an organ located in the anterior portion of the midgut. During the active stage of growth of *W. glossinidia* there are approximately 5-8 million primary symbionts in each mycetome (Aksoy, loc. cit.). It is thought that *W. glossinidia* is acquired by the offspring from milk gland secretions during larval development within its mother (Akman *et al.*, 2002). The genome of *W. glossinidia* has recently been sequenced with a size of 698 kilobases comprising 621 coding sequences (Akman *et al.*, loc. cit.). Due to the long established relationship between the tsetse fly and *W. glossinidia*, the bacteria have become adapted to life within the mycetome and have so far proved impossible to culture outside the fly (Akman *et al.*, loc. cit.). However, it has been possible to keep the symbiont metabolically active for at least 72 h under transient tissue culture conditions, thereby allowing experiments into gene expression and protein transport to take place. This study showed that the major protein product of *W. glossinidia* had a molecular mass of 60 kDa; furthermore this protein was absent from the offspring of flies that had been treated with ampicillin, confirming its bacterial origin (Aksoy, 1995b). More detailed analysis showed it was similar to heat shock proteins found in other organisms (Haines *et al.*, 2002), which are responsible for the refolding of polypeptides. Similar heat shock proteins are also over-expressed in the primary symbionts of aphids (Baumann *et al.*, 1996) and the weevil *Sitophilus oryzae* (Charles *et al.*, 1997). In many prokaryotes heat shock proteins are over-produced during times of environmental stress and the over-expression of this chaperone may be a response to life within the mycetocyte (Haines *et al.*, 2002). Expression analysis has shown that a gene coding for a transport protein involved in the uptake of ammonium is expressed at high levels (Akman and Aksoy, 2001). Ammonium produced by the fly as a nitrogenous waste product could be used as

a nitrogen source for the synthesis of amino acids by the bacterium, the products of which could be exported back to the fly.

It is thought that the primary function of *W. glossinidia* is to provide the tsetse with members of the B vitamin complex. Aposymbiotic female offspring (obtained by antibiotic treatment of pregnant females) that fed on a diet enriched with B vitamins partially regained their fecundity and subsequent experiments showed that thiamine, pantothenic acid, pyridoxine, folic acid and biotin were essential for production of pupae (Nogge, 1981). The presence of genes responsible for synthesis of these B vitamins was confirmed by the recent publication of the genome of *W. glossinidia*, supporting the suggestion that the primary function of the bacterium is to supplement the tsetse diet of vertebrate blood (Akman *et al.*, 2002).

Nogge and Ritz (1982) showed that in male tsetse both the length and (dry) weight of the mycetome continued to increase during the first 14 d of the tsetse fly's life and the ratio of mycetome (dry) weight to body weight was around 0.9 µg/mg, however, by 28 d the ratio fell to about 0.57 µg/mg. In unmated female tsetse the ratio of body weight to mycetome (dry) weight does not drop and stays at a constant level of 1 µg/mg. It takes two weeks for post-teneral growth of muscles and the hypodermis to be completed and the drop in the ratio between body weight and mycetome (dry) weight in males at around two weeks mirrors this. It has been hypothesised that the symbionts might be producing B vitamins, which aid in the development of the tsetse fly from larvae to fully-grown adult. Females retain symbionts because they need to transfer them to their offspring; this is similar to the situation in lice in which symbionts degenerate when males reach adulthood, however, in females they are retained (Ries, 1931).

### **3.1.1.2 *Sodalis glossinidius***

*Sodalis glossinidius*, the secondary endosymbiont of tsetse flies is present in all tsetse species analysed so far (Akman *et al.*, 2001). However, in wild tsetse flies not all individuals are infected; Maudlin *et al.* (1990) reported that *S. glossinidius* was present

in only 9.3% of *G. p. palpalis* examined. *S. glossinidius* is a gram-negative rod, 2-12  $\mu\text{m}$  in length, a width of 1-1.5  $\mu\text{m}$  and it is transferred to progeny through milk gland secretions during larval development (Aksoy *et al.*, 1997; Dale and Maudlin, 1999). Unlike the primary symbiont *W. glossinidia*, *S. glossinidius* is not restricted to one location within the tsetse and is found both intra- and extra-cellularly in many different tissues including the midgut, milk glands and haemolymph (Cheng and Aksoy, 1999). *S. glossinidius* was previously described as a *rickettsia* -like organism (RLO) due to the lytic zone that surrounded the bacterium within the insect cell cytoplasm (Pinnock and Hess, 1974). However, molecular characterisation of these bacteria showed that they were only distantly related to the *Rickettsiaceae* and in fact were more closely related to bacteria in the  $\gamma$  subdivision of the Proteobacteria (O'Neil *et al.*, 1993).

*S. glossinidius* was the first insect endosymbiont to be grown *in vitro*; with the use of a mosquito feeder cell culture system Welburn *et al.* (1987) successfully cultured secondary symbionts from nine tsetse species. Later Dale and Maudlin (1999) reported the culture of *S. glossinidius* on semi-defined media with N-acetyl-D-glucosamine (NAG) or raffinose as an energy source; with the tsetse fly gut being composed of chitin (polymerised NAG) it is therefore not surprising that *S. glossinidius* has adapted to use this sugar as its primary energy source (Dale and Maudlin, loc cit.).

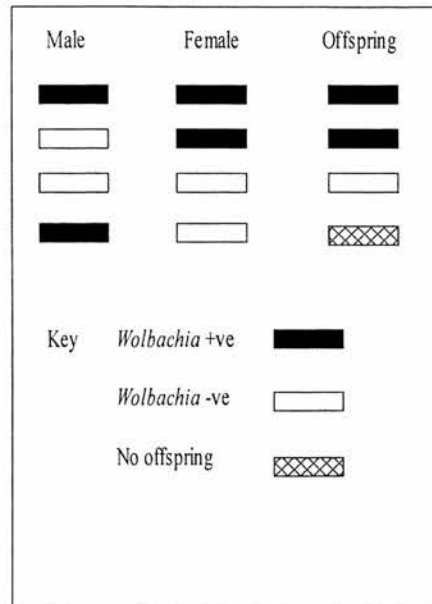
Recently Akman *et al.* (2001) using hybridisation to *E. coli* gene arrays have revealed the size of the *S. glossinidius* genome to be around 2 megabases with 1800 orthologues representing around 85% of the genome. Many of the genes found in *S. glossinidius* are geared towards the biosynthesis of amino acids and nucleic acids. Genes had previously been identified in *S. glossinidius* which did not have counterparts in *E. coli*; these were a chitinase gene (Welburn *et al.*, 1993) and a pathogenicity island containing genes for a type III secretion system (Dale *et al.*, 2001). Chitinases have been shown to be produced both *in vitro* and *in vivo* by *S. glossinidius* and are involved in the breakdown of chitin to NAG (Welburn *et al.*, 1993). Type III secretion systems are used by pathogenic bacteria to gain entry into cells and the secretion system that *S. glossinidius* possesses is similar to those found in the pathogenic bacteria *Salmonella enterica* and *Shigella* (Dale *et al.*, 2001). However, because *S. glossinidius* is found exclusively within the tsetse fly,

horizontal transfer of the pathogenicity island from other bacteria is thought to have occurred before *S. glossinidius* entered into a relationship with the tsetse fly.

### 3.1.1.3 *Wolbachia*

The third bacterium found in tsetse is similar to the parasitic *Wolbachia* found in many other insect species. The first description of *Wolbachia* was from the mosquito *Culex pipiens*, published by Hertig and Wolbach (1924). Hertig (1936) subsequently described them as a new species and genus and named them *Wolbachia pipientis* in honour of his collaborator Wolbach. *Wolbachia* is transmitted transovarially and has been estimated to affect 76% of insect species (Jeyaprakash and Hoy, 2000). It causes a variety of reproductive anomalies in insects, including parthenogenesis in parasitoid wasps (Stouthamer *et al.*, 1993), feminisation of genetic males in the wood louse *Armadillidium vulgare* (Rigaud and Jachault, 1993) and cytoplasmic incompatibility (CI) in *Drosophila* (Turelli and Hoffman, 1995) and mosquitoes (Sinkins, 2004). CI results in embryonic death due to disruptions in early fertilisation events, however, reproductive anomalies only occur when mating happens between an infected male and an uninfected female (Figure 3.1).





**Figure 3.1: *Wolbachia* mediated CI in insects.**

*Wolbachia* renders the sperm incapable of completing fertilisation successfully after entry into an uninfected egg cytoplasm. When females are infected with the same *Wolbachia* strain they somehow negate or rescue the action of *Wolbachia* on sperm and fertilisation occurs as normal. This ‘mod resc’ model involves the ‘mod’ function acting on the male germline and if the ‘resc’ function is not active in the fertilised zygote, no development occurs (Charlat *et al.*, 2001). When males and females contain different *Wolbachia* strains, CI is also observed demonstrating that ‘mod’ and ‘resc’ interact in a specific manner (Charlat *et al.*, loc cit.). It is also possible to transfer CI between insect species; Braig *et al.* (1994) transferred *Wolbachia* from the mosquito *Aedes albopictus* to *Drosophila simulans* resulting in the appearance of CI in the fruit fly.

Analysis of *Wolbachia* gene sequences from different insect hosts has shown that they are a monophyletic assemblage within the  $\alpha$  subdivision of Proteobacteria and are most closely related to the *Ehrlichia* assemblage (Cheng *et al.*, 2000). The clade containing *Wolbachia* is split into four groups (A-D): Groups A and B contain *Wolbachia* that are found in insects, mites and crustaceans; while groups C and D contain *Wolbachia* found in nematodes (Stouthamer *et al.*, 1999). It is thought that groups A and B separated from

C and D around 100 million years ago, while A and B diverged around 60 million years ago. The *Wolbachia* in groups C and D are thought to form symbiotic relations with the nematodes they infect (Taylor and Hoerauf, 1999).

The prevalence of infection with *Wolbachia* in tsetse differs between colony and wild flies; colonies of tsetse are usually 100% infected with *Wolbachia*, the exception being the *palpalis* group of flies which seem to be free of infection with *Wolbachia* (Cheng *et al.*, 2000). However, in the wild there is a completely different picture with infection rates ranging from 0-98% depending on tsetse species and location. The prevalence of *Wolbachia* infection in *G. austeni* was found to be 48% in Kenya, while in South Africa it was found to be greatly increased, with 98% of flies being infected. Populations of *G. brevipalpis* had a 34% prevalence rate of *Wolbachia* in Kenya, while in South Africa the infection rate was 0% (Cheng *et al.*, 2000). The *Wolbachia* found within *G. m. morsitans*, *G. m. centralis* and *G. brevipalpis* represent different isolates closely related within the A group of *Wolbachia* and although the *Wolbachia* isolated from *G. austeni* also belong to the A group they are distantly related to the *Wolbachia* found in the other species of tsetse flies (Cheng *et al.*, loc. cit). *Wolbachia* infections are restricted to the ovaries and testes in *G. m. morsitans* and *G. brevipalpis*, while in *G. austeni* *Wolbachia* are found in both the reproductive and somatic tissues, indicating that the different *Wolbachia* types exhibit different tissue distribution, which along with molecular evidence, suggests the *Wolbachia* found within *G. austeni* represents an independent acquisition (Cheng *et al.*, loc. cit).

CI is thought to occur in the tsetse fly but this has not been proven (O'Neill *et al.*, 1993). In tsetse there are two types of crossing incompatibility, one attributed to nuclear genes, the other attributed to maternally inherited factors (Gooding, 1990). Crosses between female *G. m. morsitans* and male *G. m. submorsitans* or *G. m. centralis* are more compatible than the reciprocal crosses (Curtis, 1972) and it is thought that *Wolbachia* might be responsible for these incompatibilities (O'Neill *et al.*, 1993).

### 3.1.2 Elimination of symbionts in insects

Antibiotics are compounds produced by bacteria which can kill or inhibit growth of other bacteria; they are useful in treating bacterial diseases of mammals and insects as they exploit differences between prokaryotic and eukaryotic cells. Common mechanisms of antibiotic action include inhibition of protein or cell wall synthesis. Protein synthesis can be blocked by a variety of mechanisms; antibiotics such as streptomycin bind to the 30S portion of the 70S ribosome and cause misreading of bacterial mRNA (Hancock, 1981; Kornder, 2002), while tetracycline attaches to the bacterial ribosome and prevents the attachment of aminoacyl-tRNA (Chopra and Roberts, 2001). Penicillin and ampicillin stop cell wall synthesis by binding to and inhibiting enzymes needed for the synthesis of the peptidoglycan cell wall (Oka and Fujita, 1978; Tipper, 1985). Eukaryotic cells possess an 80S ribosome and do not have a cell wall, hence their specificity for prokaryotes.

Antibiotics have been used successfully in *Drosophila melanogaster* to investigate CI induced by the bacterium *Wolbachia*; the addition of tetracycline to the diet removes *Wolbachia* and its associated reproductive anomalies in *D. melanogaster* (Min and Benzer, 1997).

In both aphids (Baumann *et al.*, 1995) and tsetse flies (Nogge 1976) the association between insect and primary symbiont (aphid/*Buchnera*, tsetse fly/*W. glossinidia*) is essential, as neither can survive without the other; the addition of antibiotics to the insect's diet results in slower growth and loss of reproduction in aposymbiotic offspring. Griffiths and Beck (1973) showed that treatment of aphids with antibiotics resulted in general malaise and production of non-viable offspring. The effect on tsetse is similar, the addition of antibiotics resulting in a reduction in pupal production and sterile offspring (Hill *et al.*, 1973; Nogge, 1976). However, the effects on the ant *Camponotus floridanus* and the weevil *S. oryzae* are not so apparent. Aposymbiotic carpenter ant workers were maintained for several generations without detrimental effects (Boursaux-Eude and Gross, 2000), while loss of symbionts in the weevil *S. oryzae* resulted in reduction of fitness albeit after several generations (Charles *et al.*, 1997). It is thought

that the destructive potential of the weevil is due to the presence of the symbionts which supply the weevil with vitamins increasing mitochondrial oxidative phosphorylation and enhancing the flying capacity of the insect (Heddi *et al.*, 1999). Treatment of *S. oryzae* with tetracycline over two generations is required to clear both the primary symbionts and *Wolbachia* from the insect, while heat treatment alone removes only the primary symbionts (the removal of the bacteria was confirmed by both PCR and fluorescent *in situ* hybridisation (Heddi *et al.*, 1999)).

The reduction in pupal production seen in tsetse and aphids fed antibiotics could, however, be due to toxic effects of the antibiotics, rather than the effects of the loss of the symbionts. Antibiotics commonly have side effects on eukaryotes and therefore the sterility induced could be a result of antibiotic toxicity. Mitochondria may be affected by antibiotics such as tetracycline due to the similarity of mitochondrial and bacterial ribosomes (Margulis, 1981). Harris (1967) reported that several antibiotics increased the mortality rate of the moth *Carpocapsa pomonella* while Sander (1977) showed that tetracycline produced anomalies in oogenesis and embryogenesis in the leaf hopper *Euscelis plebejus*.

### **3.1.3 Experiments to remove symbionts from tsetse**

Tsetse fly endosymbionts have been targeted by various means including complementing the bloodmeal with antibiotics (Hill *et al.*, 1973; Nogge 1976; Nogge and Gerresheim, 1982) or the addition to the bloodmeal of specific antibodies raised against the primary symbionts (Nogge, 1978; Nogge, 1980).

#### **3.1.3.1 The use of antibiotics to eliminate tsetse symbionts**

Hill *et al.* (1973) were first to investigate the effects of antibiotics on tsetse flies. Rabbits were injected with a mixture of streptomycin and benzyl penicillin (at a concentration of

15 mg/kg) 30 min before flies were allowed to feed. Morphological disturbances in symbionts were first observed after 7 d and extensive damage was noted after week 5; production of pupae was reduced from 0.45 per week at week 3 to 0.14 by week 12. Nogge (1976) used different concentrations of oxytetracycline fed through an artificial membrane; doses of 25 parts per million (ppm; 25 µg/ml) resulted in loss of pupal production but did not affect the longevity of the fly. Higher doses of oxytetracycline at concentrations of 250 ppm (250 µg/ml) and 2500 ppm (2500 µg/ml) reduced the lifespan of the flies to 18 d and 3 d respectively which was attributed to toxic effects on the flies. Nogge and Gerresheim (1982) later tested the effects of ten antibiotics and one sulphonamide on tsetse. They concluded that only three antibiotics could be given in concentrations that destroyed the symbionts and impeded host reproduction but did not affect host longevity; these were 0.05% penicillin (500 µg/ml), 0.05% kanamycin (500 µg/ml) and 0.0025% oxytetracycline (25 µg/ml). Recently, Dale and Welburn (2001) reported that the antibiotic streptozotocin (20 µg/ml) had little effect on the fertility of female tsetse flies while tetracycline (25 µg/ml) or ampicillin (50 µg/ml) both reduced the production of offspring. The F<sub>1</sub> generation was then split into two groups; the first group were infected with *T. b. rhodesiense* (strain EATRO2340) while the remaining flies were kept under standard conditions to examine their life expectancy. The streptozotocin group showed significantly lower rates of trypanosome infection than controls (27.4% compared to 43.5%). Although the trypanosome infection rates in both the tetracycline (33.3%) and ampicillin (23.8%) groups were lower than the controls, not many flies survived to dissection, which could have compromised the significance of these results. However, the offspring of streptozotocin treated flies had reduced lifespans and the authors concluded that this might be due to the toxic effects of streptozotocin rather than loss of *S. glossinidius* (Dale and Welburn, loc. cit.).

### **3.1.3.2 The use of antibodies to eliminate tsetse symbionts**

Nogge (1978) fed flies on rabbits which had been immunised against symbionts. Antibodies to the symbionts were produced by injecting a rabbit with the contents of the mycetome (50-55 mycetomes per rabbit). To act as a control, a rabbit was injected with the contents of whole flies minus the mycetome. When the concentration of antibodies to symbionts was low (1:2), pupae were still produced by the fly; however, when the concentration of antibodies was raised (1:8), pupal production ceased. Flies fed on the control rabbit showed higher rates of mortality than flies fed on the rabbit immunised with mycetome contents. Further experiments revealed that flies initially fed on low antibody titres to primary symbionts (1:2, 1:4) for 4 weeks then switched to a normal diet showed increased fecundity. This was not seen when flies were initially fed on rabbits with high antibody titres (1:32; Nogge, 1980). The use of antibodies to the primary symbionts confirmed that the reduction of pupal production was due to loss of symbionts rather than the toxic effects of the antibiotics (Nogge, loc. cit.).

### **3.1.4 Further investigations on the affects of antibiotics on tsetse flies**

In the present work further experiments were carried out to investigate the effects of streptozotocin on *G. m. morsitans*. With *S. glossinidius* linked to the susceptibility of tsetse flies to trypanosomes, the production of tsetse flies without *S. glossinidius* could illuminate the role played by the bacterium in susceptibility to infection. Tetracycline was included as it had previously been shown to stop pupal production but not affect the longevity of tsetse flies (Nogge, 1976).

### 3.1.4.1 Streptozotocin

Streptozotocin (Figure 3.2) is an N-methyl-N-nitrosourea derivative (2-deoxy-2-(3-methyl-3-nitrosoureido)-D-glucopyranoside) of NAG produced by the bacterium *Streptomyces achromogens* (Reusser, 1971; Jacobson *et al.*, 1990). It is a broad-spectrum antibiotic that also displays anti-tumour, mutagenic and diabetagenic properties (Bolzan and Bianchi 2002). Susceptible bacteria accumulate streptozotocin via the phosphoenolpyruvate dependent sugar phosphotransferase system, a membrane bound enzyme II complex encoded by the gene *nagE* (Lengeler, 1980). Streptozotocin has two effects on bacteria, initially a bacteriostatic effect caused by inhibition of PTS, resulting in depletion of the cell energy reserves followed by a bactericidal action due to phosphorylated streptozotocin breaking down, forming diazomethane a strong alkylating agent, that primarily affects replicating DNA by alkylation of cytosine residues (Jacobson *et al.*, 1990).

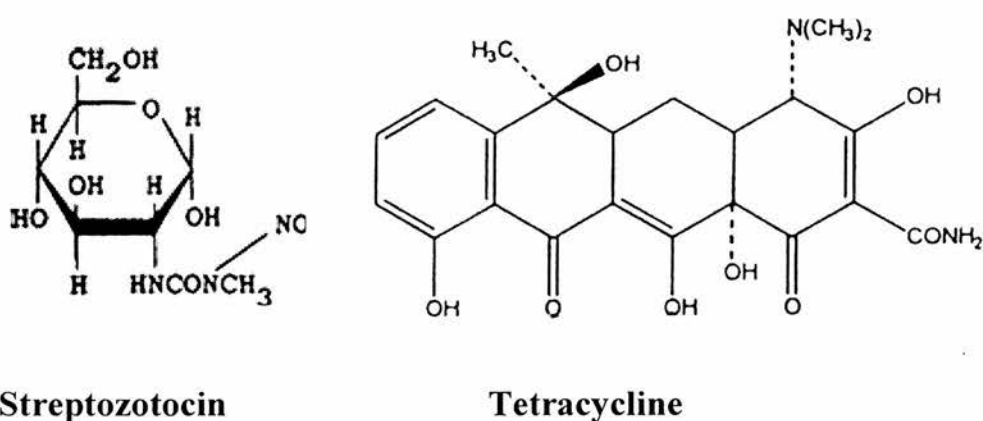


Figure 3.2: Structures of the antibiotics streptozotocin (Bolzan and Bianchi, 2002) and tetracycline (Chopra and Roberts, 2001)

### 3.1.4.2 Tetracycline

Tetracyclines are bacteriostatic antibiotics that have a broad spectrum of activity against both gram-positive and gram-negative bacteria by binding to ribosomes, thereby preventing the attachment of aminoacyl-tRNA, resulting in the inhibition of protein synthesis (Chopra and Roberts, 2001). Obtained from *Streptomyces aureofaciens* and *Streptomyces rimosus*, members of the tetracycline family were first described in the early 1940s. Tetracyclines are composed of four fused 6-membered rings to which a variety of functional groups can be attached (Figure 3.2). It is thought that tetracycline enters gram negative bacteria through the OmpF and OmpC porin channels as a positively charged cation (probably  $Mg^{2+}$ ) tetracycline complex (Schnappinger and Hillen, 1996). The tetracycline complex accumulates within the bacterial periplasm where it is thought to dissociate with the cation, allowing the uncharged tetracycline molecule to diffuse through the lipid bilayers of the bacterial cytoplasmic membrane. Once in the cell tetracycline chelates with  $Mg^{2+}$  due to both the pH and concentration of divalent metal ions being higher than outside the cell and the magnesium- tetracycline complex is thought to bind to the bacterial ribosome. The binding to the ribosome is reversible explaining the bacteriostatic effect of the tetracycline family.



3.2 Results

3.2.1 Sensitivity of *S. glossinidius* to antibiotics

See Section 2.11 for method.

The sensitivity of *S. glossinidius* to streptozotocin or tetracycline is shown in Table 3.1.

Antibiotic/concentration	25 µg	10 µg
Streptozotocin	29.5 mm (± 2.7)	23.3 mm (± .7)
Tetracycline	17 mm (± 0.6)	14.8 mm (± 1.4)

Table 3.1: Sensitivity of *S. glossinidius* to streptozotocin or tetracycline using the Kirby-Bauer disk-diffusion method.

The sensitivity of *S. glossinidius* was measured using the Kirby-Bauer disk-diffusion method (Bauer *et al.*, 1966). *S. glossinidius* proved highly susceptible to both streptozotocin and tetracycline. *S. glossinidius* also proved highly susceptible to the antibiotics augmentin, cotrimoxazole and ciprofloxacin (data not shown).

3.2.2 Larval cycle of *G. m. morsitans* fed streptozotocin or tetracycline

See Sections 2.12.1 and 2.12.2 for methods and Section 2.26.1 for details of statistical analyses.

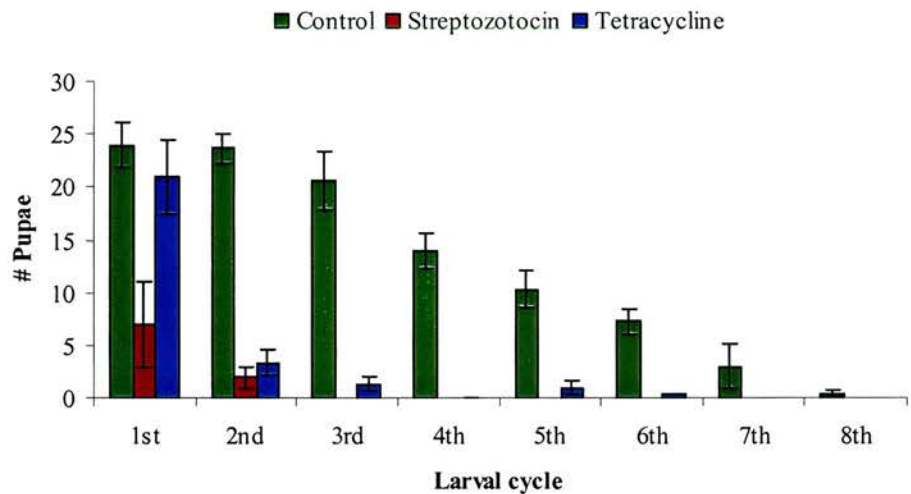
The experiments involving the addition to the bloodmeal of 25 µg streptozotocin or 50 µg tetracycline involved 95 female *G. m. morsitans* in each antibiotic group, consisting of one replicate containing 35 flies and two replicates of 30 flies. While the experiments involving the addition to the bloodmeal of 10 µg streptozotocin or 25 µg tetracycline

involved 90 female *G. m. morsitans* in each antibiotic group, consisting of three replicates of 30 flies.

The graphs for larval cycles show the average number of pupae produced per cage per larval cycle from three independent replicates. Statistical analyses were undertaken on the total number of pupae produced by the three replicates after the first larval cycle, the second larval cycle and after all larval cycles. A larvae cycle was defined as nine days from the date of deposition of the first pupae and then every nine days after that.

**3.2.2.1 Effects of feeding 25 µg/ml streptozotocin or 50 µg/ml tetracycline on the larval cycle of *G. m. morsitans***

The average number of pupae produced per cage of flies from three replicates during the eight larval cycles of female tsetse fed 25 µg/ml streptozotocin or 50 µg/ml tetracycline is shown in Figure 3.3.



**Figure 3.3: Larval cycles of *G. m. morsitans* fed 25 µg/ml streptozotocin or 50 µg/ml tetracycline. Data presented as the mean ± Standard error of mean (S.E.M) of the number of pupae deposited per cage per larval cycle from three replicates, control flies received unsupplemented bloodmeals.**

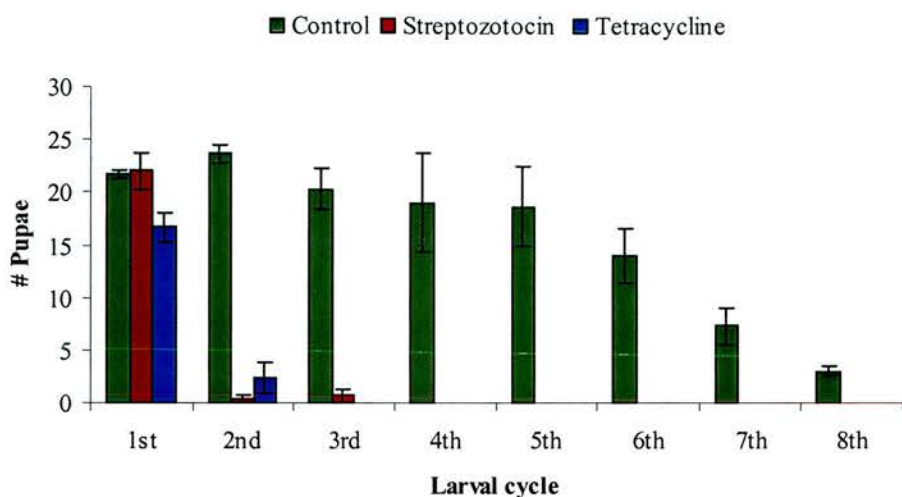
Addition of 25 µg/ml streptozotocin or 50 µg/ml tetracycline to the bloodmeal significantly reduced the number of pupae produced over eight larval cycles from a control value of 310 pupae to 27 and 81 pupae respectively (streptozotocin:  $\chi^2=279.1$ ,  $p<0.001$ ; tetracycline:  $\chi^2=143.1$   $p<0.001$ ). Control flies produced 3.3 pupae per female (ppf) while flies fed 25 µg/ml streptozotocin produced 0.3 ppf and those fed 50 µg/ml tetracycline produced 0.8 ppf.

The difference in pupae produced during the first larval cycle was significant in the streptozotocin treated group with 21 pupae being produced compared to the control value of 72, however, there was no significant difference between the control group and the tetracycline treated group which produced 63 pupae (streptozotocin:  $\chi^2=29.8$ ,  $p<0.001$ ; tetracycline:  $\chi^2=0.6$   $p=0.438$ ).

The difference in pupae produced during the second larval cycle was significant in both the 25 µg/ml streptozotocin treated group and the 50 µg/ml tetracycline treated group, reducing the amount of pupae produced from a control value of 71 pupae to 6 and 10 pupae respectively (streptozotocin:  $\chi^2=64.6$ ,  $p<0.001$ ; tetracycline:  $\chi^2=51.7$ ,  $p<0.001$ ).

#### **3.2.2.2 Effects of feeding 10 µg/ml streptozotocin or 25 µg/ml tetracycline on the larval cycle of *G. m. morsitans***

The average number of pupae produced per cage of flies from three replicates during the eight larval cycles of female tsetse fed 10 µg/ml streptozotocin or 25 µg/ml tetracycline is shown in Figure 3.4.



**Figure 3.4: Larval cycles of *G. m. morsitans* fed 10 µg/ml streptozotocin or 25 µg/ml tetracycline. Data presented as the mean ± S.E.M of the number of pupae deposited per cage per larval cycle from three replicates, control flies received unsupplemented bloodmeals.**

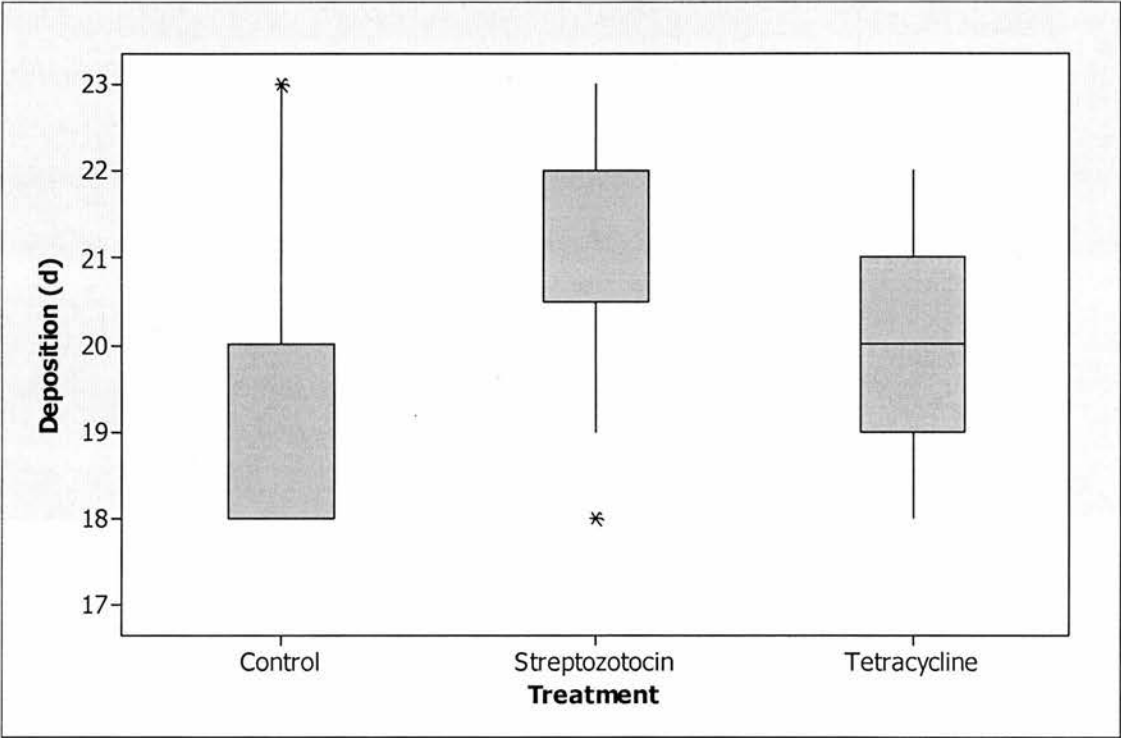
Addition of 10 µg/ml streptozotocin or 25 µg/ml tetracycline to the bloodmeal significantly reduced the number of pupae produced from a control value of 383 pupae to 69 and 57 pupae respectively (streptozotocin:  $\chi^2=240.3$ ,  $p<0.001$ ; tetracycline:  $\chi^2=270.1$   $p<0.001$ ). Control flies produced 4.3 ppf while flies fed 10 µg/ml streptozotocin produced 0.8 ppf and those fed 25 µg/ml tetracycline produced 0.6 ppf. There was no significant difference in pupae produced during the first larval cycle in either the streptozotocin treated group in which 66 pupae were produced or the tetracycline treated group where 50 pupae were produced when compared to the control value of 65 pupae (streptozotocin:  $\chi^2<0.1$ ,  $p=0.930$ ; tetracycline:  $\chi^2=2.0$ ,  $p=0.161$ ). The difference in pupae produced during the second larval cycle was significant in both the 10 µg/ml streptozotocin treated group and the 25 µg/ml tetracycline treated group, reducing the number of pupae produced from a control value of 71 pupae to 1 and 7 pupae respectively (streptozotocin:  $\chi^2=270.1$ ,  $p<0.001$ ; tetracycline:  $\chi^2=89.2$ ,  $p<0.001$ ).

**3.2.3 Deposition time of pupae from *G. m. morsitans* fed streptozotocin or tetracycline**

See Sections 2.12.1 and 2.12.2 for methods and Section 2.26.2 for details of statistical analyses.

**3.2.3.1 Effects of feeding 25 µg/ml streptozotocin or 50 µg/ml tetracycline on *G. m. morsitans* on the deposition time of the first pupae**

The time taken to deposit the first pupae of female tsetse fed 25 µg/ml streptozotocin or 50 µg/ml tetracycline is shown in Figure 3.5.

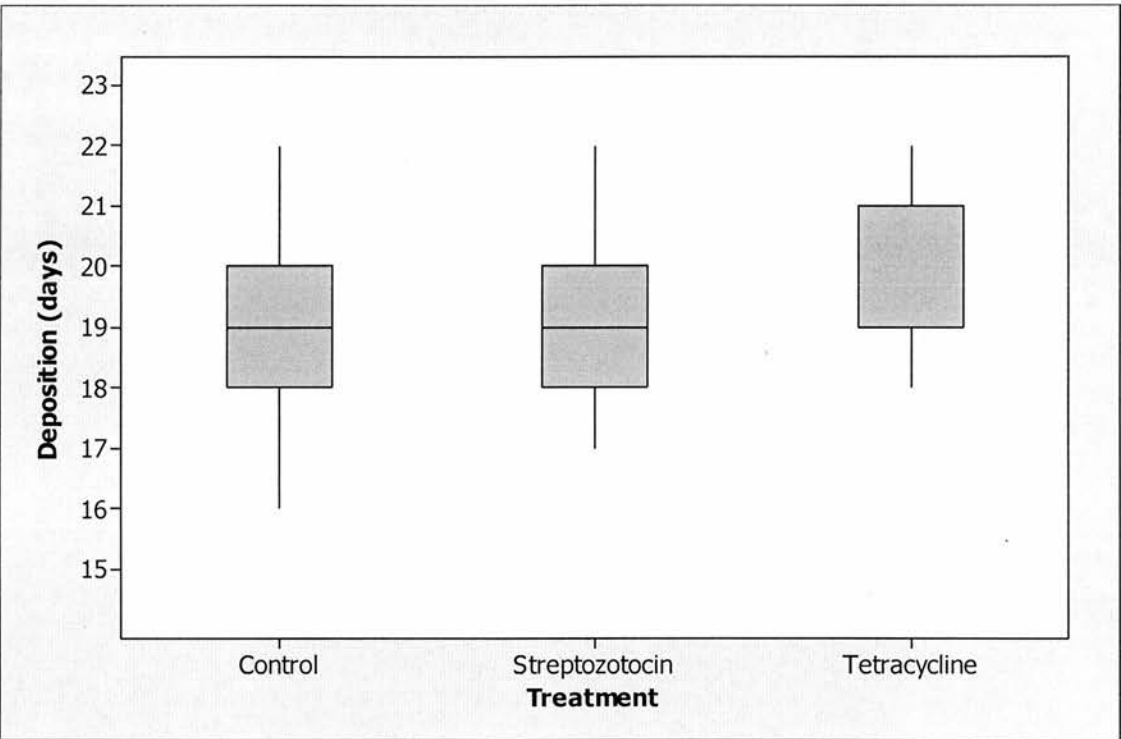


**Figure 3.5:** Time to deposit the first pupae from *G. m. morsitans* fed bloodmeals supplemented with 25 µg/ml streptozotocin or 50 µg/ml tetracycline, control flies received unsupplemented bloodmeals (\* outlier). The time to deposit the first pupae was determined by subtracting the date of mating from the date of pupal deposition.

Neither 25 µg/ml streptozotocin or 50 µg/ml tetracycline had a significant effect on the time taken to deposit the first pupae which took on average 22.4 d (n=17) and 19.7 d (n=57) respectively compared to the control value of 19.5 d (n=60; streptozotocin:  $\chi^2=1.4$ ,  $p=0.229$ ; tetracycline:  $\chi^2<0.1$   $p=0.815$ ).

**3.2.3.2 Effects of feeding 10 µg/ml streptozotocin or 25 µg/ml tetracycline on *G. m. morsitans* on the deposition time of the first pupae**

The time taken to deposit the first pupae of female tsetse fed 10 µg/ml streptozotocin or 25 µg/ml tetracycline is shown in Figure 3.6.



**Figure 3.6:** Time to deposit the first pupae from *G. m. morsitans* fed bloodmeals supplemented with 10 µg/ml streptozotocin or 25 µg/ml tetracycline, control flies received unsupplemented bloodmeals (\* outlier). The time to deposit the first pupae was determined by subtracting the date of mating from the date of pupal deposition.

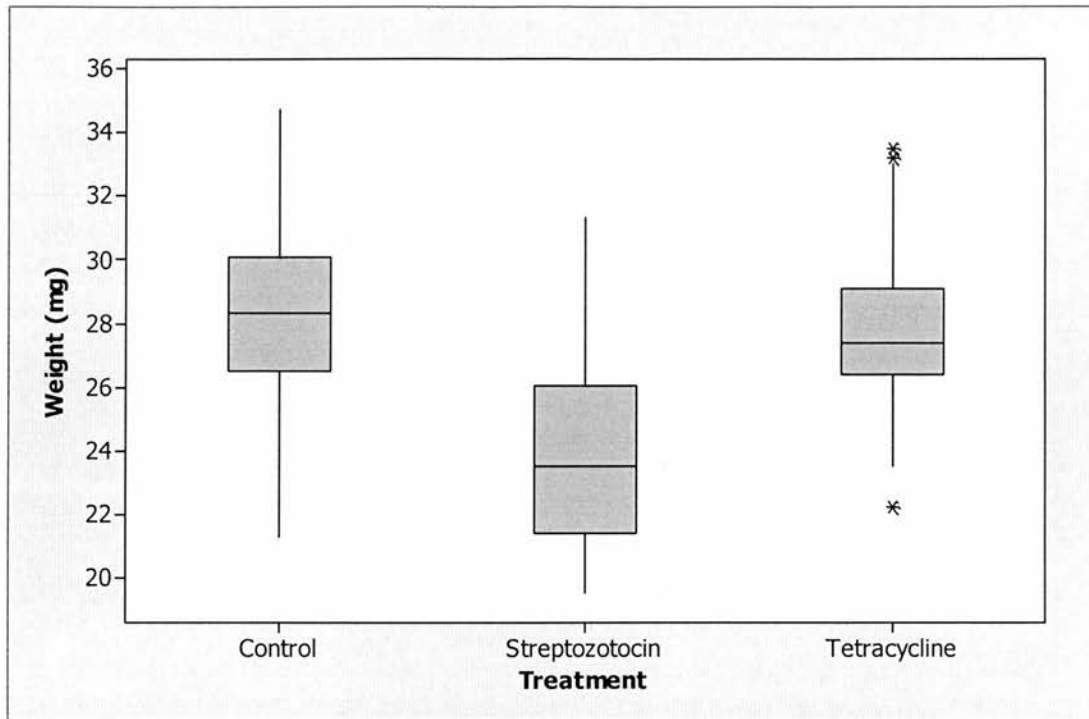
Neither 10 µg/ml streptozotocin or 25 µg/ml tetracycline had a significant effect on the time taken to deposit the first pupae which took on average 19.2 d (n=60) and 19.6 d (n=37) respectively compared to the control value of 19.3 d (n=59; streptozotocin:  $\chi^2 < 0.1$ ,  $p = 0.963$ ; tetracycline:  $\chi^2 = 0.2$ ,  $p = 0.676$ ).

### **3.2.4 Weight of pupae from *G. m. morsitans* fed streptozotocin or tetracycline**

See Sections 2.12.1 and 2.12.2 for methods and Section 2.26.3 for details of statistical analyses.

#### **3.2.4.1 Effects of feeding 25 µg/ml streptozotocin or 50 µg/ml tetracycline on the weight of the first pupae deposited by female *G. m. morsitans***

The weight of the pupae deposited during the first larval cycle of female tsetse fed 25 µg/ml streptozotocin or 50 µg/ml tetracycline is shown in Figure 3.7.



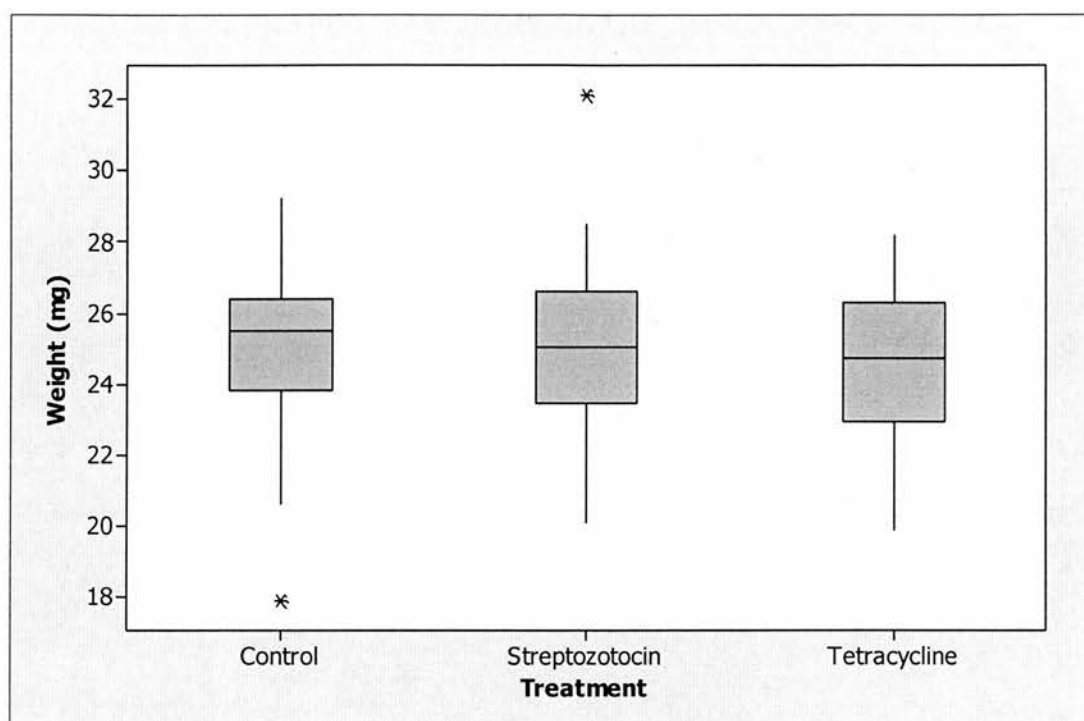
**Figure 3.7:**Weight of pupae from the first larval cycle of *G. m. morsitans* fed 25 µg/ml streptozotocin or 50 µg/ml tetracycline (\* outlier).

Addition of 25 µg/ml streptozotocin significantly reduced mean pupal weight from a control value of 28.2 mg (n=60) to 24.0 mg (n=17; F-value=27.6,  $p<0.001$ ). Those fed 50 µg/ml tetracycline produced pupae with a mean pupal weight of 27.2 mg (n=47) which were not significantly different from the controls (F-value=1.3,  $p=0.256$ ).

#### **3.2.4.2 Effects of feeding 10 µg/ml streptozotocin or 25 µg/ml tetracycline on the weight of the first pupae deposited by female *G. m. morsitans***

The weight of the pupae deposited during the first larval cycle of female tsetse fed 10 µg/ml streptozotocin or 25 µg/ml tetracycline is shown in Figure 3.8.





**Figure 3.8:** Weight of pupae from the first larval cycle of *G. m. morsitans* fed 10 µg/ml streptozotocin or 25 µg/ml tetracycline (\* outlier).

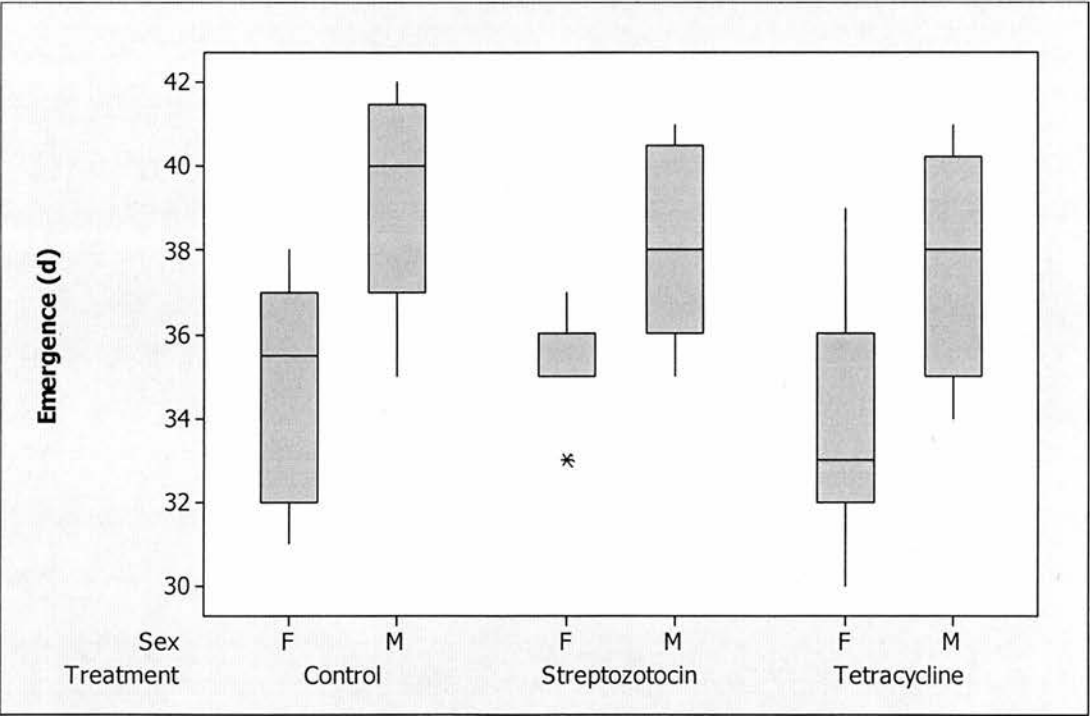
Addition of 10 µg/ml streptozotocin or 25 µg/ml tetracycline produced pupae weighing 25.0 mg (n=60) and 24.6 mg (n=37) respectively which were not significantly different from the control value of 25.0 mg (n=59; streptozotocin: F-value<0.1,  $p=0.949$ ; tetracycline: F-value=0.9,  $p=0.345$ ).

**3.2.5 Emergence time from the puparium from *G. m. morsitans* fed streptozotocin or tetracycline**

See Sections 2.12.1 and 2.12.2 for methods and Section 2.26.4 for details of statistical analyses.

**3.2.5.1 Effects of feeding 25 µg/ml streptozotocin or 50 µg/ml tetracycline on the emergence time of pupae deposited by female *G. m. morsitans***

The time taken for flies to emerge from pupae deposited during the first larval cycle of female tsetse fed 25 µg/ml streptozotocin or 50 µg/ml tetracycline is shown in Figure 3.9.



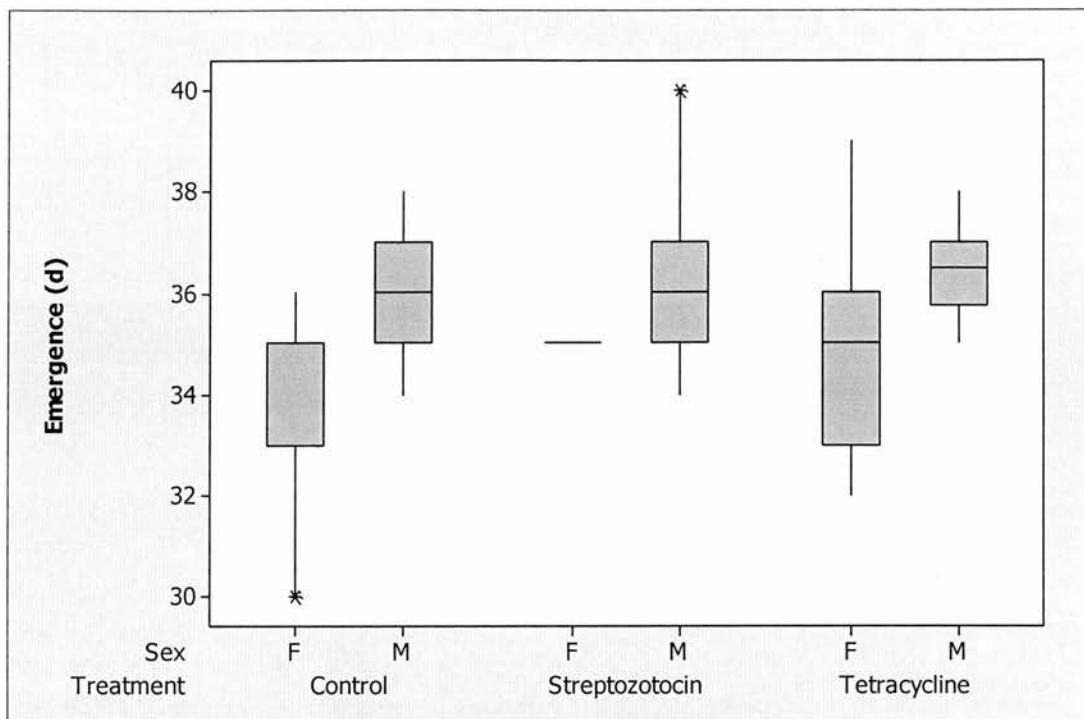
**Figure 3.9: Emergence time of females (F) and males (M) from the puparium deposited by *G. m. morsitans* fed bloodmeals supplemented with 25 µg/ml streptozotocin or 50 µg/ml tetracycline (\* outlier), control flies received unsupplemented bloodmeals. The emergence time was worked out by subtracting the date of pupal deposition from the date of emergence.**

The addition of 25 µg/ml streptozotocin or 50 µg/ml tetracycline had no significant effect on the emergence time of female tsetse which took on average 35.5 d (n=8) and 33.8 d (n=25) respectively compared to the control value of 35.5 d (n=30; streptozotocin:  $\chi^2 < 0.1$ ,  $p = 0.834$ ; tetracycline:  $\chi^2 = 0.6$ ,  $p = 0.421$ ). The addition of 25 µg/ml streptozotocin or 50 µg/ml tetracycline had no significant effect on the emergence time of male tsetse which took on average 38.1 d (n=9) and 37.9 d (n=22) respectively compared to the control value of 39.0 d (n=30; streptozotocin:  $\chi^2 = 0.2$ ,  $p = 0.618$ ; tetracycline:  $\chi^2 = 0.5$ ,  $p = 0.472$ ).

Analysis of the weight of deposited pupae from all larval cycles of the control group and the groups fed 25 µg/ml streptozotocin or 50 µg/ml tetracycline found no significant difference between the weight of pupae that developed into females or males. Pupae that developed into females or males from the control group weighed on average 28.3 mg (n=146) and 27.7 mg (n=114) respectively (F-value=1.9,  $p = 0.172$ ). Pupae that developed into females or males from the 25 µg/ml streptozotocin group weighed on average 23.5 mg (n=8) and 24.4 mg (n=9) respectively (F-value=0.3,  $p = 0.611$ ). Pupae that developed into females or males from the 50 µg/ml tetracycline group weighed on average 27.7 mg (n=27) and 27.3 mg (n=24) respectively (F-value=0.2,  $p = 0.655$ ).

### **3.2.5.2 Effects of feeding 10 µg/ml streptozotocin or 25 µg/ml tetracycline on *G. m. morsitans* on the emergence time of deposited pupae**

The time taken for flies to emerge from pupae deposited during the first larval cycle of female tsetse fed 10 µg/ml streptozotocin or 25 µg/ml tetracycline is shown in Figure 3.10.



**Figure 3.10: Emergence time of females (F) and males (M) from the puparium deposited by *G. m. morsitans* fed bloodmeals supplemented with 10 µg/ml streptozotocin or 25 µg/ml tetracycline (\* outlier), control flies received unsupplemented bloodmeals. The emergence time was worked out by subtracting the date of pupal deposition from the date of emergence.**

The addition of 10 µg/ml streptozotocin or 25 µg/ml tetracycline had no significant effect on the emergence time of female tsetse which took on average 34.9 d (n=30) and 34.7 d (n=19) respectively compared to the control value of 34.1 d (n=30; streptozotocin:  $\chi^2=0.2$ ,  $p=0.592$ ; tetracycline:  $\chi^2=0.2$ ,  $p=0.635$ ). The addition of 10 µg/ml streptozotocin or 25 µg/ml tetracycline had no significant effect on the emergence time of male tsetse which took on average 36.0 d (n=30) and 36.4 d (n=18) respectively compared to the control value of 36.1 d (n=29; streptozotocin:  $\chi^2<0.1$ ,  $p=0.983$ ; tetracycline:  $\chi^2<0.1$ ,  $p=0.887$ ).

Analysis of the weight of deposited pupae from all larval cycles of the control group and the groups fed 10 µg/ml streptozotocin or 25 µg/ml tetracycline found no significant difference between the weight of pupae that developed into females or males. Pupae that

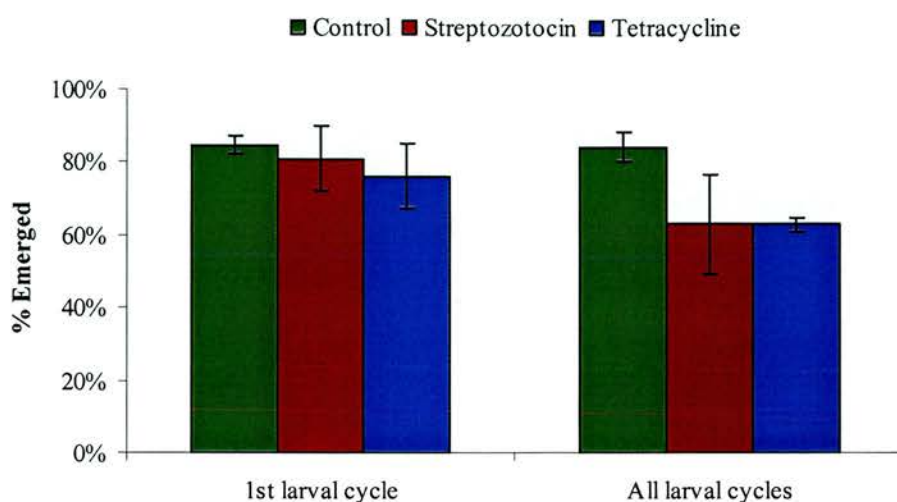
developed into females or males from the control group weighed on average 26.7 mg (n=163) and 26.1 mg (n=157) respectively (F-value=3.0,  $p=0.084$ ). Pupae that developed into females or males from the 10 µg/ml streptozotocin group weighed on average 25.0 mg (n=32) and 25.0 mg (n=29) respectively (F-value<0.1,  $p=0.902$ ). Pupae that developed into females or males from the 25 µg/ml tetracycline group weighed on average 24.6 mg (n=20) and 24.3 mg (n=19) respectively (F-value=0.2,  $p=0.633$ ).

### **3.2.6 Emergence of pupae deposited by *G. m. morsitans* fed streptozotocin or tetracycline**

See Sections 2.12.1 and 2.12.2 for methods and Section 2.26.5 for details of statistical analyses.

#### **3.2.6.1 Effects of feeding 25 µg/ml streptozotocin or 50 µg/ml tetracycline on the percentage emergence of pupae deposited by female *G. m. morsitans***

The percentage of flies to emerge from pupae deposited during the first larval cycle and all larval cycles of female tsetse fed 25 µg/ml streptozotocin or 50 µg/ml tetracycline is shown in Figure 3.11.



**Figure 3.11: Percentage of flies emerging from pupae deposited during the first larval cycle or all larval cycles of flies fed bloodmeals supplemented with either 25 µg/ml streptozotocin or 50 µg/ml tetracycline. Data presented as the mean ± S.E.M of the number of flies successfully emerging from the puparium from three replicates, control flies received unsupplemented bloodmeals.**

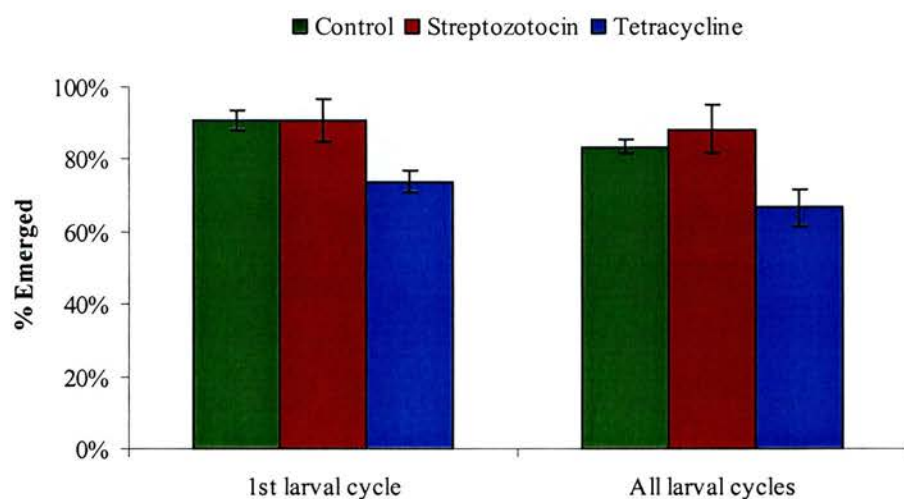
The addition of 25 µg/ml streptozotocin or 50 µg/ml tetracycline had no significant effect on emergence of the pupae deposited during the first larval cycle, emergence rates were 80% (n=21) and 75% (n=62) respectively compared to the control value of 85% (n=71; streptozotocin:  $\chi^2 < 0.1$ ,  $p = 0.872$ ; tetracycline:  $\chi^2 = 1.9$ ,  $p = 0.172$ ). However, analysis of all pupae deposited during all larval cycles showed a significant decrease in emergence of pupae from a control value of 84% (n=310) to 63% in both the streptozotocin (n=27) and tetracycline (n=81) treated groups (streptozotocin:  $\chi^2 = 8.4$ ,  $p = 0.015$ ; tetracycline:  $\chi^2 = 16.1$ ,  $p < 0.001$ ).

Failure to emerge was linked to reduced pupal weight at deposition in both control flies and those fed either 25 µg/ml streptozotocin or 50 µg/ml tetracycline. Analysis of pupal weights from control flies deposited from all larval cycles showed that there was a significant decrease from the control value of 28.0 mg to 26.3 mg (F-value=11.5,  $p < 0.001$ ). Analysis of pupal weights from flies fed 25 µg/ml streptozotocin deposited from all larval cycles showed that there was a significant decrease from the emerged

value of 24.0 mg (n=17) to the non-emerged value of 15.8 mg (n=10; F-value=34.1,  $p<0.001$ ). Analysis of pupal weights from flies fed 50 µg/ml tetracycline deposited from all larval cycles showed that there was a significant decrease from the emerged value of 27.3 mg (n=51) to the non-emerged value of 23.0 mg (n=30; F-value=34.1,  $p<0.001$ ).

### 3.2.6.2 Effects of feeding 10 µg/ml streptozotocin or 25 µg/ml tetracycline on the percentage emergence of pupae deposited by female *G. m. morsitans*

The percentage of flies to emerge from pupae deposited during the first larval cycle and all larval cycles of female tsetse fed 10 µg/ml streptozotocin or 25 µg/ml tetracycline is shown in Figure 3.12.



**Figure 3.12:** Percentage of flies emerging from pupae deposited during the first larval cycle or all larval cycles of flies fed bloodmeals supplemented with either 10 µg/ml streptozotocin or 25 µg/ml tetracycline. Data presented as the mean  $\pm$  S.E.M of the number of flies successfully emerging from the puparium from three replicates, control flies received unsupplemented bloodmeals.

Addition of 25 µg/ml tetracycline significantly reduced emergence from pupae deposited during the first larval cycle from a control value of 91% (n=65) to 74% (n=50;  $\chi^2=5.8$ ,  $p=0.016$ ). This difference was also significant when pupae from all larval cycles were



analysed with tetracycline significantly reducing emergence rates from a control value of 84% (n=383) to 67% (n=57;  $\chi^2=8.2$ ,  $p=0.004$ ). Addition of 10  $\mu\text{g/ml}$  streptozotocin had no significant effect on emergence of pupae deposited during the first larval cycle with 91% (n=66) of pupae emerging successfully ( $\chi^2<0.1$ ,  $p=0.978$ ) or from all larval cycles where 91% (n=69) of pupae emerged ( $\chi^2=1.9$ ,  $p=0.169$ ).

Failure to emerge was linked to reduced pupal weight at deposition in control flies and those fed 25  $\mu\text{g/ml}$  tetracycline. Analysis of pupal weights from control flies deposited from all larval cycles showed that there was a significant decrease from the emerged value of 28.0 mg (n=320) to the non-emerged value of 26.3 mg (n=63; F-value=6.8,  $p=0.010$ ). Analysis of pupal weights from flies fed 25  $\mu\text{g/ml}$  tetracycline deposited from all larval cycles showed that there was a significant decrease from the emerged value of 24.5 mg (n=38) to the non-emerged value of 22.2 mg (n=19; F-value=5.7,  $p=0.020$ ). There was no significant difference between the weights of emerged and non-emerged pupae from flies fed 10  $\mu\text{g/ml}$  streptozotocin which were 25.0 mg (n=61) and 24.6 mg (n=7) respectively (F-value=0.2,  $p<0.664$ ).

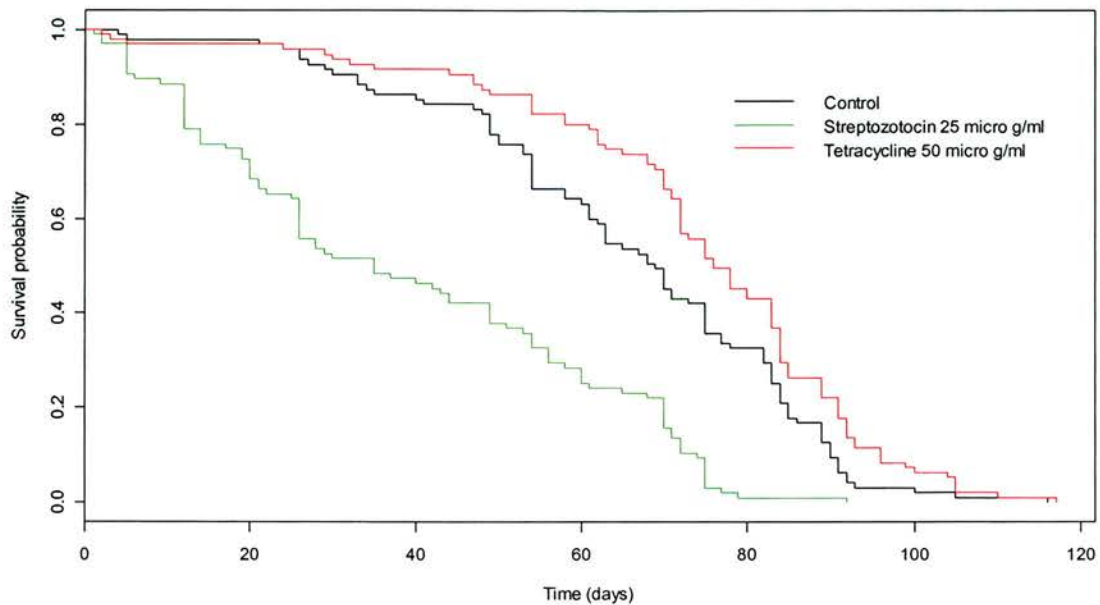
### **3.2.7 Survival of *G. m. morsitans* fed streptozotocin or tetracycline**

See sections 2.12.1 and 2.12.2 for methods and section 2.26.6 for details of statistical analyses.

#### **3.2.7.1 Effects of feeding 25 $\mu\text{g/ml}$ streptozotocin or 50 $\mu\text{g/ml}$ tetracycline on the survival of *G. m. morsitans***

Survival analyses of female flies fed 25  $\mu\text{g/ml}$  streptozotocin or 50  $\mu\text{g/ml}$  tetracycline are shown in Figure 3.13.



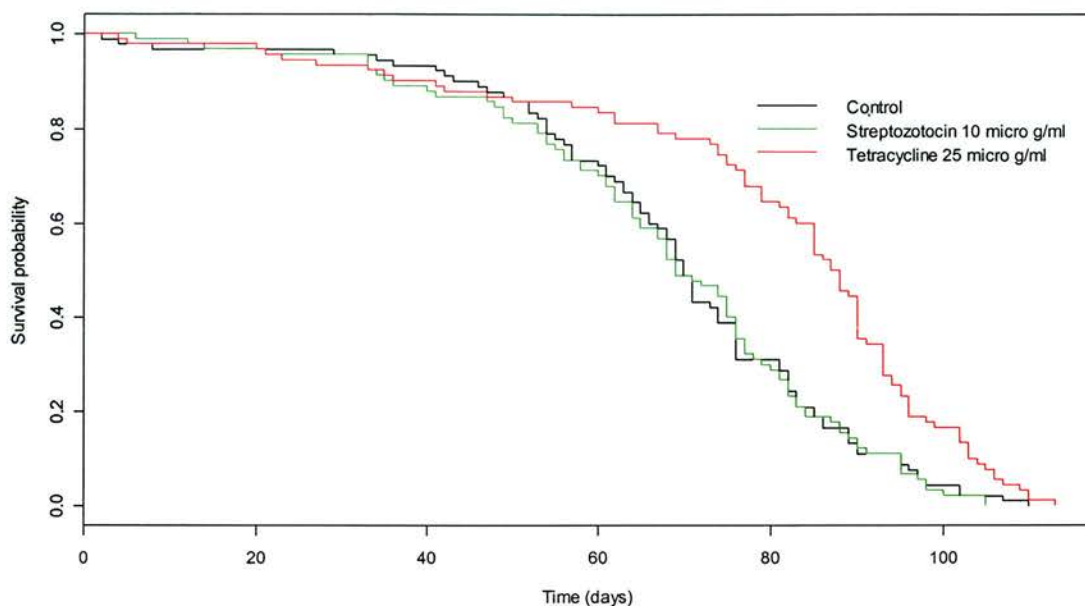


**Figure 3.13: Survival analyses of *G. m. morsitans* fed 25 µg/ml streptozotocin or 50 µg/ml tetracycline, control flies received unsupplemented bloodmeals. The lifespan of each individual fly was worked out by subtracting the date of death of each individual fly from the date of the start of the experiment.**

Addition of 25 µg/ml streptozotocin to the bloodmeal significantly reduced the lifespan of tsetse from a control value of 65.5 d to 39.1 d ( $\chi^2=46.1$ ,  $p<0.001$ ). Addition of 50 µg/ml tetracycline to the bloodmeal significantly increased lifespan to 73.5 d ( $\chi^2=7.1$ ,  $p=0.008$ ).

### **3.2.7.2 Effects of feeding 10 µg/ml streptozotocin or 25 µg/ml tetracycline on the survival of *G. m. morsitans***

Survival analyses of female flies fed 10 µg/ml streptozotocin or 25 µg/ml tetracycline are shown in Figure 3.14.



**Figure 3.14: Survival analyses of *G. m. morsitans* fed 10 µg/ml streptozotocin or 25 µg/ml tetracycline, control flies received unsupplemented bloodmeals. The lifespan of each individual fly was worked out by subtracting the date of death of each individual fly from the date of the start of the experiment.**

Addition of 10 µg/ml streptozotocin to the bloodmeal had no significant effect on lifespan of the tsetse, which on average lived 67.7 d compared to the control value of 68.4 d ( $\chi^2=0.1$ ,  $p=0.734$ ). Addition of 25 µg/ml tetracycline to the bloodmeal significantly increased the lifespan of the tsetse flies to 79.9 d ( $\chi^2=20.6$ ,  $p<0.001$ ).

### **3.2.8 PCR analyses of the offspring of *G. m. morsitans* fed streptozotocin or tetracycline for symbionts**

See Sections 2.12.1 and 2.12.2 for methods, 2.13 for isolation of DNA from tsetse flies and 2.14 for PCR parameters.

### 3.2.8.1 Effects of feeding 25 µg/ml streptozotocin or 50 µg/ml tetracycline on the symbiont status of *G. m. morsitans*

PCR analyses of flies for symbiont status is summarised in Table 3.2.

	Insect	<i>W. glossinidia</i>	<i>S. glossinidius</i>	<i>Wolbachia</i>
Control	100%	100%	100%	100%
Streptozotocin 25 µg/ ml	100%	100%	0%	100%
Tetracycline 50 µg/ml	100%	0%	0%	100%

**Table 3.2: PCR analyses of offspring of flies treated with 25 µg/ml streptozotocin or 50 µg/ml tetracycline, control flies received unsupplemented bloodmeals. Table shows the percentage of flies positive for DNA by PCR of *W. glossinidia*, *S. glossinidius* and *Wolbachia*. PCR for insect DNA was included as a template control.**

Addition of 25 µg/ml streptozotocin to the bloodmeal selectively removed *S. glossinidius* from the offspring of treated flies from a control value of 100% (n=20) to 0% (n=10), however it had no effect on either *W. glossinidia* or *Wolbachia* inheritance. Addition of 50 µg/ml tetracycline removed both *W. glossinidia* and *S. glossinidius* from the offspring of treated flies from a control value of 100% (n=20) to 0% (n=20), however, it had no effect on *Wolbachia* inheritance.

### 3.2.8.2 Effects of feeding 10 µg/ml streptozotocin or 25 µg/ml tetracycline on the symbiont status of *G. m. morsitans*

PCR analyses of flies for symbiont status is summarised in

Table 3.3.

	Insect	<i>W. glossinidia</i>	<i>S. glossinidius</i>	<i>Wolbachia</i>
Control	100%	100%	100%	100%
Streptozotocin 10 µg/ ml	100%	100%	0%	100%
Tetracycline 25 µg/ml	100%	0%	0%	100%

**Table 3.3: PCR analyses of offspring of flies treated with 10 µg/ml streptozotocin or 25 µg/ml tetracycline, control flies received unsupplemented bloodmeals. Table shows the percentage of flies positive for DNA by PCR of *W. glossinidia*, *S. glossinidius* and *Wolbachia*. PCR for insect DNA was included as a template control.**

Addition of 10 µg/ml streptozotocin to the bloodmeal selectively removed *S. glossinidius* from the offspring of treated flies from a control value of 100% (n=20) to 0% (n=20), however it had no effect on either *W. glossinidia* or *Wolbachia* inheritance. Addition of 25 µg/ml tetracycline removed both *W. glossinidia* and *S. glossinidius* from the offspring of treated flies from a control value of 100% (n=20) to 0% (n=20), however, it had no effect on *Wolbachia* inheritance.

### **3.2.9 Sequencing of PCR products obtained from *W. glossinidia* and *Wolbachia* specific primer sets**

See section 2.15 for details on sequencing of PCR products.

The PCR products obtained from amplification of 170F/1227R and 81F/691R were sequenced and found to be for the *W. glossinidia* 16S ribosomal gene (accession number AF022876) from *G. m. morsitans* Score =696 bits (351), Expect =0.0) and *wsp* gene

(accession number AF020079) from *G. m. morsitans* respectively (Score=1103 bits (556), Expect =0.0).

### **3.2.10 The use of streptozotocin to create a line of flies without *S. glossinidius***

See Section 2.12.3 for methods, Section 2.26 for details on statistical analyses, Section 2.13 for DNA extraction and Section 2.14 for PCR analyses.

#### **3.2.10.1 Effects of feeding 10 µg/ml or 5 µg/ml streptozotocin on the puparial production of female *G. m. morsitans***

Of the 40 flies that started the experiment 38, 39 and 33 remained alive when the flies were separated into individual cages on day 14 in the control, 5 µg/ml streptozotocin and 10 µg/ml streptozotocin treated groups respectively.

The control group produced 25 pupae during the first larval cycle which was not significantly different from either the streptozotocin 5 µg/ml or the streptozotocin 10 µg/ml groups which produced 21 and 19 pupae respectively (5 µg/ml:  $\chi^2=0.2$ ,  $p=0.638$ ; 10 µg/ml:  $\chi^2=0.5$ ,  $p=0.467$ ). In total 83 pupae were deposited (2.1 ppf) from the control group which was significantly more than the streptozotocin 5 µg/ml group (0.53 ppf) and the streptozotocin 10 µg/ml group (0.48 ppf) which did not produce any more pupae after the first larval cycle (5 µg/ml:  $\chi^2=20.5$ ,  $p<0.001$ ; 10 µg/ml:  $\chi^2=18.1$ ,  $p<0.001$ ).

#### **3.2.10.2 Effects of feeding 10 µg/ml or 5 µg/ml streptozotocin on the deposition time of the first pupae by female *G. m. morsitans***

There was no significant difference between the time taken for female flies to deposit pupae during the first larval cycle in either the streptozotocin 5 µg/ml (n=21) or the streptozotocin 10 µg/ml (n=19) groups taking 16.2 and 16.5 d respectively compared to

the control (n=25) value of 16.5 d (5 µg/ml:  $\chi^2 < 0.1$ ,  $p = 0.836$ ; 10 µg/ml:  $\chi^2 < 0.1$ ,  $p = 0.968$ )

#### **3.2.10.3 Effects of feeding 10 µg/ml or 5 µg/ml streptozotocin on the emergence time of pupae deposited by female *G. m. morsitans***

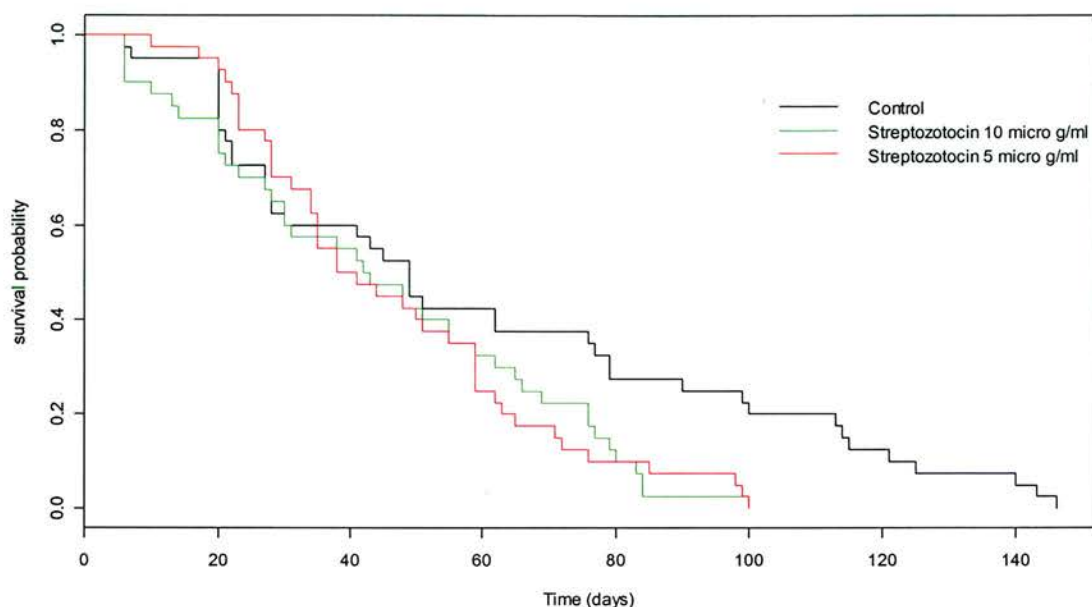
There was no significant difference between the time taken for female flies to emerge in either the streptozotocin 5 µg/ml or the streptozotocin 10 µg/ml groups taking 27.0 (n=11) or 27.3 d (n=10) respectively compared to the control value of 27.0 d (n=8; 5 µg/ml:  $\chi^2 < 0.1$ ,  $p > 0.999$ ; 10 µg/ml:  $\chi^2 < 0.1$ ,  $p = 0.903$ ). There was no significant difference between the time taken for male flies to emerge in either the streptozotocin 5 µg/ml or the streptozotocin 10 µg/ml groups taking 30.2 (n=8) or 30.1 d (n=7) respectively compared to the control value of 29.8 d (n=13; 5 µg/ml:  $\chi^2 < 0.1$ ,  $p = 0.870$ ; 10 µg/ml:  $\chi^2 < 0.1$ ,  $p = 0.908$ ).

#### **3.2.10.4 Effects of feeding 10 µg/ml or 5 µg/ml streptozotocin on the percentage emergence of pupae deposited by female *G. m. morsitans***

There was no significant difference in emergence rates with 90% (n=21) and 89% (n=19) flies emerging from the streptozotocin 5 µg/ml or streptozotocin 10 µg/ml groups respectively compared to the control value of 84% (n=25; 5 µg/ml:  $\chi^2 = 0.4$ ,  $p = 0.516$ ; 10 µg/ml:  $\chi^2 = 0.3$ ,  $p = 0.600$ ).

#### **3.2.10.5 Effects of feeding 10 µg/ml or 5 µg/ml streptozotocin on the survival of *G. m. morsitans***

Survival analyses of female flies fed 10 µg/ml or 5 µg/ml streptozotocin is shown in Figure 3.15.



**Figure 3.15: Survival analyses of *G. m. morsitans* fed 10 µg/ml or 5 µg/ml streptozotocin, control flies received unsupplemented bloodmeals. The lifespan of each individual fly was worked out by subtracting the date of death of each individual fly from the date of the start of the experiment.**

Addition of either 5 µg/ml or 10 µg/ml streptozotocin significantly reduced survival of tsetse from a control value of 60 d to 47 d and 44 d respectively (5 µg/ml streptozotocin:  $\chi^2=5.4$ ,  $p=0.021$ ; 10 µg/ml streptozotocin:  $\chi^2=4.7$ ,  $p=0.031$ ).

### 3.2.10.6 The F<sub>1</sub> and F<sub>2</sub> generation from *G. m. morsitans* fed streptozotocin

In the F<sub>1</sub> generation the control group of flies produced 8 females and 13 males; four of these females were mated and two of those went on to produce offspring. The first female (life span 95 d) produced one pupa while the second female (lifespan 83 d) produced four pupae. The average lifespan of the females were 69 d and 17 d for mated and non-mated flies respectively while male flies lived on average 27 d. The streptozotocin (5 µg/ml) treated group produced 11 females and 10 males. Six of these

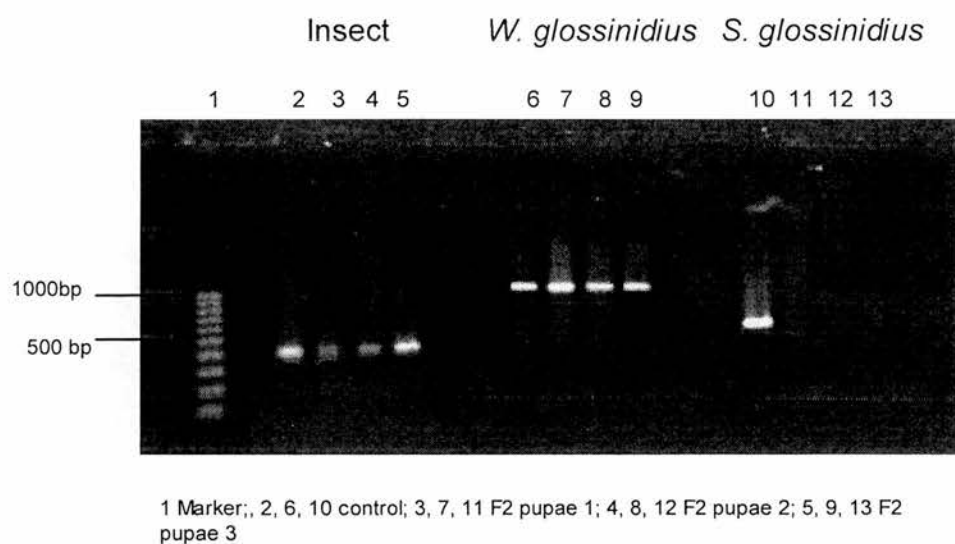


females were mated and one went on to produce offspring. This female lived for 61 d and produced four offspring. Three of the other mated females did not survive past 5 d due to their inability to take a bloodmeal. The average lifespan of the females were 69 d and 17 d for mated and non-mated flies respectively while male flies lived on average 28 d. The streptozotocin (10 µg/ml) treated group produced 10 females and 7 males. Five of these females were mated and one went on to produce offspring. This female lived for 85 d and produced four offspring. Two of the other mated females did not survive past 5 d due to their inability to take a bloodmeal. The average lifespan of the females were 69 d and 17 d for mated and non-mated flies respectively while male flies lived on average 26 d. Significant differences in survival times were found between control mated females and streptozotocin (5 µg/ml) mated females ( $\chi^2=4.6$ ,  $p=0.032$ ) and between control unmated females and streptozotocin (10 µg/ml) unmated females ( $\chi^2=4.4$ ,  $p=0.035$ ). No significant differences were found between unmated control females and unmated streptozotocin females (5 µg/ml;  $\chi^2=1.9$ ,  $p=0.166$ ) and between mated control females and mated streptozotocin females (10 µg/ml;  $\chi^2=1.5$ ,  $p=0.220$ ). There were no significant differences between control males and males from the offspring fed streptozotocin at either 5 µg/ml or 10 µg/ml doses (5 µg/ml:  $\chi^2=0.1$ ,  $p=0.774$ ; 10 µg/ml:  $\chi^2=0.1$ ,  $p=0.797$ ).

### 3.2.10.7 PCR analysis of F<sub>2</sub> pupae

PCR analyses of F<sub>2</sub> pupae deposited from females treated with 10 µg/ml streptozotocin are shown in Figure 3.16.





**Figure 3.16: PCR analyses of F<sub>2</sub> pupae for symbiont status.** Three pupae from the F<sub>2</sub> generation of flies fed 10 µg/ml streptozotocin were analysed for possession of *W. glossinidia* and *S. glossinidius*. A pupa from the F<sub>2</sub> generation from flies fed unsupplemented bloodmeals was included as a control while PCR for insect DNA was included as a template control.

Three of the F<sub>2</sub> pupae that were deposited from streptozotocin 10 µg/ml group were analysed by PCR for *W. glossinidia* and *S. glossinidius*, standard insect primers were included as control. There was no amplification for *S. glossinidius* DNA while all pupae were positive for *W. glossinidia* DNA and tsetse DNA.

### 3.3 Discussion

As in previous experiments the addition of antibiotics had a significant effect on tsetse reproduction (Hill *et al.*, 1973; Nogge, 1976). The addition of the antibiotics streptozotocin or tetracycline resulted in almost complete cessation of pupal production after the first larval cycle. However, although pupal production was significantly reduced by the addition of tetracycline, interestingly, significant increases in the lifespans of the treated tsetse flies were observed. The addition of 10 µg/ml streptozotocin did not significantly affect lifespan of tsetse but did stop pupal deposition after one larval cycle.

#### 3.3.1 Susceptibility of *S. glossinidius* to antibiotics

Both streptozotocin and tetracycline proved highly effective at reducing the growth of *S. glossinidius* grown on cell free medium. It is not surprising that streptozotocin had this effect on *S. glossinidius* as streptozotocin enters cells using the same transport system that is responsible for the uptake of NAG (Lengeler, 1980). It is unlikely that streptozotocin resistance would develop in *S. glossinidius* as its primary energy source is NAG (Dale and Maudlin, 1999) and as resistance to streptozotocin is linked to loss of the phosphoenolpyruvate-dependent sugar phosphotransferase system in *E. coli* (Lengeler, 1980), it is likely that this would be a lethal mutation in *S. glossinidius*. Tetracycline was not as effective as streptozotocin in inhibiting the growth of *S. glossinidius*. Resistance to tetracycline in commensal and pathogenic bacteria is a relatively recent phenomenon; analyses of members of *Enterobacteriaceae* collected before the widespread use of tetracycline for clinical, veterinary and agricultural purposes show very low levels of resistance (Hughes and Datta, 1983). Tetracycline resistance has, however, risen dramatically since its introduction due to the acquisition of *tet* genes commonly found on mobile units such as plasmids (Mendez *et al.*, 1980;

Chopra and Roberts, 2001). The *tet* genes have spread between bacterial species by conjugation and the same plasmid responsible for resistance to tetracycline has been found in several unrelated bacterial species (Chopra and Roberts, 2001). It would be unlikely that *S. glossinidius* would possess genes responsible for resistance to tetracycline; although *S. glossinidius* in theory could come into contact with bacteria possessing *tet* genes when the fly takes a bloodmeal, there would be no selective advantage for the bacteria to show resistance to tetracycline.

The greater efficacy of streptozotocin compared to tetracycline seen in the present work may be due to its bactericidal properties; although initially showing bacteriostatic action due to inhibition of the sugar phosphotransferase system, streptozotocin breaks down to form the bactericidal diazomethane a potent DNA alkylating agent (Lengeler, 1980). Tetracycline is a bacteriostatic antibiotic (Chopra and Roberts, 2001) and this may explain why it is not as efficient as streptozotocin at inhibiting the growth of *S. glossinidius*.

### 3.3.2 Offspring production

Due to the viviparous means of reproduction of tsetse, analysis of deposited pupae is a good indicator of the fitness status of the mother (Moloo and Kutuza, 1985). Although there were no significant affect of tetracycline or the lower doses of streptozotocin (10 µg/ml or 5 µg/ml) on the number of pupae deposited during the first larval cycle, addition of either streptozotocin or tetracycline virtually stopped pupal production after the first larval cycle. Previous studies have not fully reported the number of pupae that were deposited during experiments. Hill *et al.* (1973) reported that feeding tsetse on rabbits injected with antibiotics reduced the number of pupae produced per week from 0.45 per week at week three to 0.14 at week 12, but did not detail the number of pupae produced from tsetse fed on rabbits that were not treated with antibiotics. Nogge (1976) and Nogge and Gerresheim (1982) reported only that oxytetracycline inhibited pupal production and did not give any other information except that the tsetse produced “very

little or not at all” (Nogge and Gerresheim, loc. cit.). In the current work total pupae production was reduced from a control value of 3.3 ppf to 0.3 ppf and 0.8 ppf for those fed 25 µg/ml streptozotocin or 50 µg/ml tetracycline respectively and from a control value of 4.3 ppf to 0.8 ppf and 0.6 ppf for those fed 10 µg/ml streptozotocin and 25 µg/ml tetracycline respectively. The current results with streptozotocin contrast with work of Dale and Welburn (2001) who reported that streptozotocin had no significant impact on pupal production. Although Dale and Welburn (loc. cit.) used a streptozotocin concentration of 20 µg/ml (resulting in 3.1 ppf compared to the control value of 3.6 ppf), in the present study lower concentrations (10 µg/ml and 5 µg/ml) blocked pupal production after the first larval cycle. The number of pupae produced per female when tetracycline was added to the bloodmeal was, however, similar to the number reported by Dale and Welburn (2001) who found that tsetse fed tetracycline produced 0.7 ppf. Streptozotocin has been reported to be toxic to insect germ cells (Hernando *et al.*, 2004), suggesting that it could damage oocytes or sperm stored in the spermatheca. To test if streptozotocin was toxic to sperm or oocytes, female tsetse were dissected and the reproductive cells were examined; fully motile sperm were observed in the spermatheca and no obvious damage was noted to the oocytes (data not shown), suggesting that streptozotocin was not overtly toxic to the reproductive cells. However, further work on a larger scale needs to be carried out to fully examine if streptozotocin has a toxic effect on the germ cells of tsetse. The primary mode of action of streptozotocin is the alkylation of cytosine residues leading to DNA damage which could have other effects on male and female germ cells which were not apparent or the antibiotic could affect the fertilisation process. No abortions were observed in the flies treated with streptozotocin, suggesting that the antibiotic either affects the fertilisation process or an event leading to fertilisation.

### **3.3.3 Time taken to deposit the first pupae**

Although the time taken to deposit larvae was three days longer in flies that had been fed 25 µg/ml streptozotocin the difference was not significant. However, the low number of deposited larvae from the flies fed 25 µg/ml streptozotocin could have compromised the significance of this result. All other treatments resulted in the average deposition of larvae between 19 and 20 d showing that either dose of tetracycline (25 µg/ml or 50 µg/ml) or the lower dose of streptozotocin (10 µg/ml) had no effect on deposition time. Previous experiments with tsetse fed antibiotics have made no mention of deposition time and so cannot be compared with the current work.

### **3.3.4 Weight of the first pupae deposited**

Only streptozotocin at a concentration of 25 µg/ml had a significant effect on the pupal weight, although, as only 17 of the 27 pupae that were deposited emerged, this could have compromised the significance of these results. However, the most likely cause of the reduction in pupal weight would be the toxic effect of the antibiotic on the fly, as 25 µg/ml streptozotocin significantly reduced the lifespan of tsetse (while 10 µg/ml did not have an effect on either the pupal weight or lifespan). Although tetracycline at concentrations of either 50 µg/ml or 25 µg/ml did not significantly affect pupal weight during the first larval cycle, pupae deposited during subsequent larval cycles did have lower birth weights, however, most of these pupae failed to emerge. Previous work with tsetse fed antibiotics has not included pupal weights, however, analysis by Birkenmeyer and Dame (1975), who were investigating if the weight of deposited pupae could be used to sex the fly, found no significant difference between the weight of deposited female or male pupae, which agrees with the current work.

### **3.3.5 Emergence from the puparium**

No significant effects due to antibiotic treatment were found in the time taken to emerge from the puparium. This suggests that in the flies that emerged antibiotics were either not toxic to the developing larvae or that the pupae do not contain high enough levels of antibiotic to affect their development. However, 25 µg/ml streptozotocin and tetracycline at concentrations of either 50 µg/ml or 25 µg/ml significantly decreased the percentage of flies emerging from deposited pupae, suggesting that the pupae that did not emerge could have been damaged by the antibiotics. Dale and Welburn (2001) reported that tetracycline at concentration of 25 µg/ml reduced emergence from a control value of 94% to 33%. In the current work the same dose also reduced the emergence rate although not to the same extent, with 67% of treated pupae emerging compared to the control value of 84%. Streptozotocin at a concentration of 10 µg/ml had no significant effect on emergence rates; however, at the higher concentration of 25 µg/ml, emergence rates from the puparium were significantly decreased. Dale and Welburn (*loc. cit.*) found that 20 µg/ml streptozotocin reduced emergence rates from a control value of 94% to 79%.

### **3.3.6 Lifespan**

Addition of 25 µg/ml streptozotocin to the bloodmeal significantly reduced the lifespan of mated female tsetse, suggesting that at this concentration the antibiotic is toxic. At a concentration of 10 µg/ml streptozotocin contrasting results were obtained; in the first experiment where flies were kept together, no significant difference was found between the control and treated groups but in the experiment where flies were separated and kept individually the difference between the treated groups and control groups was significant. However, this difference was of borderline significance and may have been a result of the chilling involved to separate the flies into individual cages. Previous

experiments by Dale and Welburn (2001) had shown that addition of streptozotocin reduced the lifespan of the F<sub>1</sub> generation; however, no mention was made if the lifespan of the flies fed streptozotocin was affected. It is interesting that addition of tetracycline significantly increased the lifespan of mated tsetse. In previous experiments, high concentrations of oxytetracycline of 250 µg/ml or 2500 µg/ml resulted in the death of all flies after 18 d and 3 d respectively (Nogge and Gerresheim, 1982). However, full details of the lifespan were not given with the data presented by Nogge and Gerresheim (loc. cit.) in the form of 50% survival of flies on day X (X being the number of days from the start of the experiment when 50% of the flies were alive) and 0% survival on day X. Consequently the data for flies fed 25 µg/ml oxytetracycline was given as 80 d for 50% survival compared to the control value of 95 d and >100 d for 0% survival in both control flies and those that were treated with oxytetracycline. Nogge and Gerresheim (loc. cit.) reported 0% survival of >100 d, so it is unclear if 25 µg/ml oxytetracycline had a significant effect on lifespan. However, during the current work 25 µg/ml tetracycline increased the 50% survival rate from a control value of 70 d to 87 d which could suggest that oxytetracycline is more toxic to tsetse than tetracycline. The production of offspring can have a detrimental effect on the lifespan of the parent; for example *Drosophila* bred for increased longevity show a decreased reproductive capacity (Rose, 1984; Rose, 1999). Since tetracycline stops larval production in the tsetse fly, it is possible that nutrients normally used for egg production will instead be used by other tissues in the fly. The *Loi de Balancement* idea proposed by Geoffroy Saint-Hilaire (1818) suggested that “The atrophy of one organ turns to the profit of another; and the reason why this cannot be otherwise is simple, it is because there is not an unlimited supply of the substance required for each part”. Thus the metabolic savings made by not producing offspring could have led to the increased longevity seen in tsetse fed tetracycline. The fact that streptozotocin did not increase longevity but did reduce larval production suggests that it could have toxic effects on tsetse as shown in other eukaryotic cells (Weiss, 1982). The increased lifespan of flies treated with tetracycline could also be due to the antibacterial action of the antibiotic. Although care was taken to keep the collection of blood as sterile as possible, it is possible that bacterial



contamination could be cause of death in control tsetse. Tetracycline is a broad spectrum antibiotic active against both gram-positive and gram-negative bacteria, however, streptozotocin is only active against cells utilising PTS, giving it a lower range of action (Lengeler, 1980). The greater antibacterial range of tetracycline compared to streptozotocin could account for the increased lifespan seen in flies fed tetracycline.

### **3.3.7 Detection of symbiotic bacteria by PCR**

PCR analysis showed that streptozotocin selectively cleared *S. glossinidius* from the offspring of treated flies. Addition of the antibiotic tetracycline to the tsetse diet cleared both *W. glossinidia* and *S. glossinidius*; however, elimination of *Wolbachia* was not possible. As streptozotocin is particularly effective against bacteria utilising PTS it is not surprising that *S. glossinidius* was removed from the offspring of treated flies. The gene *nagE* is responsible for the transport system in which streptozotocin is taken into the cell and both *W. glossinidius* and *S. glossinidius* have been shown to carry the *nagE* gene (Akman and Aksoy, 2001; Akman *et al.*, 2001). *S. glossinidius* has been shown to use NAG as its primary energy source, however, the primary energy source of *W. glossinidia* has not been determined but early experiments had shown that pyruvate, malate and succinate were the main substrates for respiration (Wink, 1979). Although *Wolbachia* DNA was detected in the offspring of flies fed tetracycline it could be possible that the PCR was detecting DNA from dead bacteria as *Wolbachia* is found within female germ cells and is transmitted transovarially.

### **3.3.8 Attempts to establish a *S. glossinidius*-free colony of *G. m. morsitans***

Experiments have shown that presence of *S. glossinidius* within tsetse flies is linked to susceptibility to trypanosome infection (Maudlin and Dukes, 1985; Maudlin *et al.*, 1986). Maudlin *et al.* (1990) found a positive correlation between trypanosome infection



and *S. glossinidius* presence within a wild population of *G. p. palpalis* in Liberia. Therefore the removal of *S. glossinidius* from the tsetse could shed some light on why some flies show susceptibility to infection and why others are refractory. Dale and Welburn (2001) found that the offspring of flies fed streptozotocin produced significantly fewer midgut infections when challenged with a standard trypanosome infected bloodmeal. In the present work preliminary studies had shown it was possible to obtain F<sub>2</sub> pupae which did not possess *S. glossinidius*, so further experiments were set up to try and obtain enough flies to establish a colony. Three attempts were made to establish a line of flies which had had *S. glossinidius* removed by streptozotocin treatment. However, these experiments failed to produce sufficient offspring to produce a viable colony. This was probably due to streptozotocin treatment stopping larval production after one larval cycle. Although some insects such as aphids can take as little as 6 d to complete a generation (Lamb, 1992), it takes at least six weeks to obtain an emerged tsetse from a treated parent; this long generation time as well as the small number of pupae that were produced resulted in failure to produce a *S. glossinidius* negative line of flies. Although Maudlin *et al.* (1986) produced a colony of trypanosome susceptible flies from a single mother, this took three years and such an extensive time frame was too long for the current investigation.

Currently one of the methods being examined to reduce the burden of trypanosomiasis is genetic engineering of *S. glossinidius* to produce trypanocidal compounds which will affect the vectorial capacity of the tsetse (Aksoy *et al.*, 2001). This has been successful in *R. prolixus* where the symbiotic bacterium *Rhodococcus rhodnii* has been transformed to produce cecropins (Beard *et al.*, 1998). In these bugs juvenile *R. prolixus* obtain their symbiotic bacteria by ingesting adult faecal material contaminated with bacteria.

However, in tsetse both *W. glossinidia* and *S. glossinidius* are inherited through milk gland secretions (Chen and Aksoy, 1999; Akman *et al.*, 2002) and if transformed *S. glossinidius* was to be used as a carrier of anti-trypanosomal compounds it is likely that the resident population of *S. glossinidius* would have to be replaced. The most likely method to remove *S. glossinidius* would be the use of antibiotics and it may be possible

using lower concentrations of streptozotocin, to clear *S. glossinidius* without having a negative effect on the reproduction of the fly.

### **3.3.9 Conclusions**

The importance of symbionts for reproduction in tsetse is clear as the present work shows that the addition of antibiotics drastically reduced the number of pupae produced. However, symbionts seem not to be required for the tsetse itself to function; indeed addition of tetracycline to the bloodmeal resulted in increased longevity. As the present work failed to create successfully a line of flies deficient in *S. glossinidius*, no experiments could be carried out to examine the role of this symbiont in susceptibility to trypanosome infection.

## **Chapter 4**

# **Factors affecting midgut infection rates of trypanosomes in tsetse flies**

## 4.1 Introduction

The vast majority of tsetse flies are refractory to infection with trypanosomes of the *Trypanozoon* and *Nannomonas* groups. Both these groups of trypanosomes must establish an infection in the midgut of the tsetse before maturing and forming mammalian infective metacyclics in the salivary glands and the proboscis respectively (Hoare, 1972). As *T. vivax* completes its life cycle exclusively within the proboscis of the tsetse fly it does not need to establish an infection in the midgut, and therefore tsetse may pick up infections at any point during their lives (Woolhouse and Hargrove, 1998).

### 4.1.1 Factors that influence midgut infections

Several factors have been shown to be important in the susceptibility of the fly to infection. These include temperature, age of fly at the time of infection, host factors, fly/trypanosome combinations and variation between individual flies (Molyneux, 1977; Maudlin, 1991; Molyneux and Stiles, 1991).

#### 4.1.1.1 Temperature

Temperature has been shown to play a role in both influencing susceptibility to trypanosome midgut infection and controlling the subsequent maturation of midgut forms. Ndegwa *et al.* (1992) investigated the effect of temperature on trypanosome infection rates by incubating pupae of *G. m. centralis*, *G. f. fuscipes* and *G. brevipalpis* at 25°C or at 28°C during daytime and at 25°C at night. Incubation at the higher temperature significantly increased midgut infection rates of *T. congolense* in all three tsetse species. Lowering the puparial incubation temperature to 22°C resulted in a significant decrease of midgut infections rates when the emerged flies were returned to

25°C and infected compared to pupae continually kept at 25°C (Welburn and Maudlin, 1991). Analysis of pupae kept at 22°C showed a reduction in hybridisation of a DNA probe to *Sodalis glossinidius* and it was suggested that the numbers of *S. glossinidius* was positively correlated with susceptibility to infection (Welburn and Maudlin, loc. cit.).

#### **4.1.1.2 Fly age**

The timing of exposure to an infective feed has been shown to play an important role in the development of midgut infections. Otieno *et al.* (1983) found that flies that fed on an infective bloodmeal less than 8 h after emergence produced higher levels of midgut infections than flies fed 12-24 h after emergence. It has been suggested that an incomplete peritrophic membrane (PM) makes younger flies easier to infect than older flies which have a fully formed PM (Ellis and Evans, 1977; Otieno *et al.*, 1983; Lehane and Msangi, 1991). This hypothesis was shown to be incorrect as the addition of glucosamine to the infective feed of both teneral and non-teneral flies raised midgut infections to similar levels, suggesting that a fully formed PM is not a barrier to infection (Welburn and Maudlin, 1992). However, the most important age factor in susceptibility to infection is prior exposure to a bloodmeal; clearly tsetse flies which have fed on an uninfected bloodmeal are more refractory to infection than those which have not fed (teneral state). This was first recognised by Van hoof *et al.* (1937) and subsequent experiments by Wijers (1957) and Welburn and Maudlin (1992) confirmed the decreased susceptibility to infection of fed flies. Welburn and Maudlin (loc. cit) obtained midgut infections rates of 57% compared to 20% in non-teneral flies.

#### 4.1.1.3 Fly/ trypanosome species

Experiments have shown that flies of the *morsitans* group are the most susceptible to infection followed by the *fusca* group and the *palpalis* group. Moloo and Kutuza (1988a) compared the susceptibility to infection of seven different tsetse species to two stocks of *T. b. brucei*. *G. m. centralis* was the most susceptible producing midgut infection rates of 81% when infected with a *T. b. brucei* stock from Tanzania compared with 59% in *G. brevipalpis*. Midgut infection in the *palpalis* group of flies (*G. p. palpalis*, *G. p. gambiense*, *G. f. fuscipes* and *G. tachinoides*) and *G. austeni* were rare, with trypanosomes being found in only 3-6% of flies. Infection with a Nigerian strain of *T. b. brucei* produced similar results, *G. m. centralis* and *G. brevipalpis* producing midgut infection rates of 36% and 18% respectively, while the *palpalis* group and *G. austeni* produced infection rates of <2% (Moloo and Kutuza, loc. cit.). Although *G. austeni* is a member of the *morsitans* group, it behaves as a member of the *palpalis* group in relationship to trypanosome infection. *G. austeni* has been considered a discrete sister clade of the *morsitans* group (Chen *et al.*, 1999) and therefore would be expected to differ in its biochemical properties. Similar infection rates were produced when flies were challenged with *T. congolense* with *G. m. centralis* being the most susceptible, followed by *G. brevipalpis* and flies of the *palpalis* group and *G. austeni* showing the lowest infection rates (Moloo and Kutuza, 1988b)

#### 4.1.1.4 Host factors

Several host factors have been shown to be involved in the establishment of midgut infections. Tsetse flies infected with goat and pig blood generally result in higher midgut infection rates than those fed on waterbuck and eland. Mihok *et al.* (1993) found that *G. m. morsitans* fed on blood from goats, pigs, buffalo, waterbuck, eland and cow produced midgut infection rates of 29%, 18%, 11%, 9%, 7% and 5% respectively. The individual

components of blood also can affect subsequent trypanosome infections. Serum plays an important role in blocking the establishment of midgut infections; both Gingrich *et al.* (1982) and Maudlin *et al.* (1984) found that removal of serum from the infective bloodmeal resulted in significant increases in trypanosome infections. Red blood cells have also been showed to be important in the transformation of trypanosomes from bloodstream forms to procyclics (Nguu *et al.*, 1996). Further experiments showed that no transformation occurred in plasma from both rat or eland blood and that it was the rat red blood cells that promoted transformation (Nguu *et al.*, loc. cit.).

#### **4.1.1.5 Variation between individual flies**

Susceptibility to infection has been linked to the presence of the maternally inherited bacterium *S. glossinidius* (Maudlin, 1982; Maudlin *et al.*, 1986; Dale and Maudlin; 1999). *S. glossinidius* was thought to influence infection rates by breaking down chitin to N-acetyl-D-glucosamine (NAG) during pupation which it then uses as an energy source (Welburn *et al.*, 1993). The glucosamine produced is thought to interfere with the action of tsetse midgut lectins normally responsible for trypanosome killing (Maudlin and Welburn, 1987). Susceptible lines of tsetse have been created and susceptibility to trypanosome infection has been shown to persist over at least 13 generations (Maudlin and Dukes, 1985). However, susceptible tsetse flies which have previously fed show similar infection rates to wild type tsetse suggesting that maternally inherited susceptibility to trypanosome infection is a phenomenon limited to teneral flies (Welburn and Maudlin, 1992). A positive correlation between *S. glossinidius* and presence of trypanosome midgut infections has been shown in natural populations; Maudlin *et al.* (1990) found that *G. nigrofusca*, considered the most effective vector of trypanosomiasis in Liberia, had a high prevalence of *S. glossinidius* while *G. p. palpalis*, highly refractory to trypanosome infection, showed low levels of bacteria.

### 4.1.2 Immune responses of insects

Insects lack a specific immune response and rely on an innate immune response which is also found in vertebrates. Recently it has become apparent that many of the signalling pathways involved in recognition of foreign invaders in insects are shared with mammals (Hoffman, 2002; Naitza and Ligoxygakis, 2004). Signalling in response to pattern recognition of foreign invaders in insects is through a membrane receptor encoded by the gene *Toll* which activates Rel factors Dorsal and Dif (Hultmark, 2003). Dorsal is also involved in dorsal-ventral polarity during embryo development (Anderson *et al.*, 1984) while Dif is involved in activating immune responses (Ip *et al.*, 1993), a process involving many signalling factors closely related to interleukin-1 and Toll-like receptor pathways in humans (Hultmark, 2003). In recent years there has been a lot of work investigating the *Drosophila* immune response which consists of three components: a humoral response, a cellular response and a phenoloxidase reaction (Hultmark 2003). Lectins (Franc and White, 2000) and free radicals (Foley and O'Farrell, 2003) have also been shown to play a role in insect immunity.

#### 4.1.2.1 Humoral immune response

The humoral response of insects is mediated by the production of antimicrobial peptides; so far 34 peptides belonging to 8 families have been found in the *Drosophila* genome (Hultmark, 2003). These include the drosomycins and metchnikowins that are active against fungi, the defensins active against gram-positive bacteria and the attacins, cecropins, dipterocins and drosocins which are active against gram-negative bacteria. A humoral factor has been found in tsetse haemolymph which significantly reduces the motility of trypanosomes (East *et al.*, 1983). Although *T. brucei* has been observed in tsetse haemolymph (Mshelbwala, 1972; Otieno, 1973), it is thought that invasion of the haemolymph is not involved in the life cycle of the trypanosome as factors in the haemolymph would rapidly kill any trypanosomes which enter (East *et al.*, 1983).



Recently the peptides defensin, dipterecin and attacin have been shown to be encoded in the tsetse fly (Hao *et al*, 2001). Under normal conditions tsetse flies produce high levels of dipterecin, low levels of defensin while attacin is undetectable. In response to a trypanosome infected bloodmeal, expression of defensin and attacin in the fat body begin to rise during the transformation of the bloodstream to the procyclic form. After 20 d both defensin and attacin are transcribed at high levels in flies with midgut infections; flies which have cleared infections show lowered levels of transcription (Hao *et al*, 2001).

#### **4.1.2.2 Cellular immune response**

Cellular immune responses involve two pathways: phagocytosis and the encapsulation of foreign bodies. The only phagocytic cell found in adult *Drosophila* is the plasmatocyte; phagocytosis begins with the attachment of the plasmatocyte to the foreign body, followed by internalisation and destruction of the invader in the phagosome, the process being very similar to vertebrate macrophages (Meister, 2004). If the foreign body is too large to be phagocytosed the insect uses encapsulation which involves the complete encasement of the invader. The process starts with the binding of plasmatocytes to the foreign object and the production in the fat body of lamellocytes which then encase the foreign body in a multilayered capsule (Meister, 2004). Death of the invading organism follows due to lack of oxygen or the production of cytotoxic free radicals.

Studies in tsetse have shown at least three different haemocytes are present (East *et al*, 1980). Type 1 and Type 2 cells make up over 95% and 2% of the total haemocyte population and are both thought to be plasmatocytes. Type 3 haemocytes are small spherical cells making up 2% of the population and are similar to prohaemocytes.

#### 4.1.2.3 Phenoloxidase response

The phenoloxidase response results in melanotic encapsulation and is activated by foreign bodies including beta-1, 3-glucans, lipopolysaccharides and peptidoglycans (Cerenius and Soderhall, 2004). The enzyme prophenoloxidase exists in an inactive state in the haemolymph until triggered, resulting in the production of quinones from the oxygenation of monophenols and melanin which encapsulates invaders. A phenoloxidase response has been characterised in tsetse haemolymph and differences have been shown between refractory *G. p. palpalis* and susceptible *G. m. morsitans*. Using established *in vitro* techniques Nigam *et al.* (1997) showed that addition of *T. b. rhodesiense* to the haemolymph from *G. p. palpalis* resulted in higher levels of activation of prophenoloxidase than haemolymph from *G. m. morsitans*. Differences in activation of prophenoloxidase were also found between male and female *G. m. morsitans* and it was suggested that the phenoloxidase response could play a role in maturation.

#### 4.1.2.4 Lectin response

Lectins are found in a wide variety of plants, animals and bacteria and are cell-agglutinating proteins of non-immune origin possessing the ability to bind with free sugars, or with the sugar residues of polysaccharides, glycoproteins and glycolipids (Sharon, 1977). The binding of a lectin to a sugar is as precise as an enzyme to its substrate, or an antibody to an antigen. Binding of lectins can cause agglutination of cells and they were originally called phytohaemagglutinins due to their ability to bind red blood cells. Lectins are thought to play an important immune role in several insects including tsetse flies (Maudlin and Welburn, 1987; Welburn *et al.*, 1989), sand flies (Wallbanks *et al.*, 1986) and *R. prolixus* (Pereira *et al.*, 1981). *In vitro* experiments with the plant lectin concanavalin A showed that binding of the lectin to procyclic trypanosomes results in the death of the cell in a process similar to

apoptosis (Welburn *et al.*, 1996). The apoptotic pathway was first discovered in 1972 and is a form of programmed cell death where the cell receives a signal to die (Kerr *et al.*, 1972). In eukaryotes the cell undergoes a series of events which results in the activation of caspases resulting in DNA fragmentation and membrane blebbing. Within the last ten years apoptotic pathways have been found in several unicellular eukaryotes, these include *T. b. rhodesiense*, (Welburn *et al.* 1996) *T. cruzi* (Ameisen *et al.*, 1995), *Leishmania* spp. (Chulay *et al.*, 2001) and *Plasmodium* (Al-Olayan *et al.*, 2001). Apoptotic pathways generally start with the binding of effector molecules to the surface of cells resulting in the production of secondary messengers signalling the cell to die. *In vitro* three lectins have been shown to induce this effect in both *T. brucei* and *T. congolense* procyclics: concanavalin A, wheat germ agglutinin and *Ricinus communis* agglutinin, all specific for branched mannoses (Pearson *et al.*, 2000). The lectins had no effect on bloodstream form trypanosomes expressing VSG, showing that the lectins are specific to the surface coat of procyclics. The addition of procyclin to the infective bloodmeal of tsetse flies increases infection rates in similar ways to those obtained with glucosamine suggesting the midgut lectins are specific to procyclin and that blocking these lectins increases the likelihood of an infection (Welburn and Maudlin, 1999). The first report of hemagglutination and trypanosome agglutination in tsetse was published by Ibrahim *et al.* (1984). Extracts of *G. austeni* midguts and hindguts were specifically inhibited by D-glucosamine and caused the agglutination of procyclic *T. brucei*. Experiments by Maudlin and Welburn (1987) showed that extracts from midguts of tsetse flies bred for susceptibility to trypanosome infection showed significantly less erythrocyte agglutinating activity than flies bred for refractoriness to infection. The addition of D+ glucosamine to the infective bloodmeal resulted in a significant increase in susceptibility to infection. Maudlin and Welburn (1987) fed *G. m. morsitans* doses of D+ glucosamine ranging from 7.5 mM to 60 mM, producing midgut infections rates of nearly 100%. Mihok *et al.* (1992) reported that supplementation of the bloodmeal with D-glucosamine for 5 d significantly increased susceptibility to *T. b. brucei* in *G. m. morsitans*, *G. m. centralis* and *G. pallidipes*. However, D+ glucosamine was toxic to the tsetse flies with significant deaths occurring in all three species.

It was hypothesised that during pupation there is a build up of D+ glucosamine within the midgut and when the tsetse emerges to take its first bloodmeal from an infected animal, the sugars produced by *S. glossinidius* bind and inhibit the midgut lectins. After the first feed, the inhibitory sugars are excreted, explaining why the teneral tsetse is more susceptible to trypanosome infection (Welburn and Maudlin, 1992).

Several investigations have shown that *G. p. palpalis* is more refractory to infection than *G. m. morsitans* (Moloo and Kutuza, 1988a; Welburn *et al.*, 1994). While the addition of D+ glucosamine to the infective bloodmeal of *G. m. morsitans* results in midgut infection rates of 100%, in *G. p. palpalis*, the addition of D+ glucosamine does not raise infection rates over 60% (Welburn *et al.*, 1994). *G. p. palpalis* and *G. pallidipes* have been shown to possess two trypanocidal lectins: a glucosyl lectin which can be inhibited by D+ glucosamine and a galactosyl molecule inhibited by D+ galactose and both sugars are required to promote the superinfection levels seen in *G. m. morsitans* (Welburn *et al.*, 1994). The glucosyl lectin binds to rabbit erythrocytes and is present in guts of fed *G. m. morsitans* and *G. p. palpalis*. The galactosyl lectin does not show any erythrocyte binding capacity, however, a midgut trypanolysin is found in *G. p. palpalis* but not in *G. m. morsitans* (Stiles *et al.*, 1990). *G. tachinoides*, a close relative of *G. p. palpalis*, has been found to possess two midgut lectins, comprising of 26 and 29 kDa components. (Grubhoffer *et al.*, 1994). The possession of two trypanocidal lectins was thought to account for the increased refractoriness of the *palpalis* group to trypanosome infection.

#### 4.1.2.5 Free radicals

In recent years free radicals have been shown to play a role in defence against a variety of vector borne pathogens in the insects *Anopheles stephensi* and *Rhodnius prolixus*. Luckhart *et al.* (1998) showed that nitric oxide (NO) played an important role in stopping the development of malaria parasites in the mosquito *A. stephensi*. The addition to the diet of L-arginine, the substrate of nitric oxide (NO) significantly decreased infection rates while the addition of L-NAME a nitric oxide synthase inhibitor (NOS)

significantly increased infection rates. Superoxide and NO have been shown to play a role in the defence of the *R. prolixus* against the parasite *Trypanosoma rangeli*. *T. rangeli* invades the haemolymph of its vector as it makes its way to the salivary glands and elevated levels of both superoxide and NO have been reported in *R. prolixus* in response to injection into the haemolymph of *T. rangeli* parasites (Whitten *et al.*, 2001). Addition of a NOS inhibitor resulted in high number of deaths of insects in response to infection, suggesting that NO usually plays a role in regulating infection (Whitten *et al.*, loc. cit.). NO has also been shown to play an important role in the immune response of *Drosophila* to infection with gram negative bacteria (Foley and O'Farrell, 2003). Tsetse fed on a bloodmeal containing trypanosomes show both reduced levels of NO and activity of NOS compared to flies fed on uninfected blood (Hao *et al.*, 2003). However, the addition to the infective bloodmeal of either L-arginine or L-NAME had no effect on midgut infection rates suggesting that NO does not play a role in the outcome of midgut trypanosome infections (Hao *et al.*, 2003).

#### **4.1.3 Cellular stress**

A fine balance usually exists between oxidants and antioxidants in a cell. Oxidative stress can result from an increase in the levels of oxidants or a decrease in the concentration of antioxidants of the cell. The production of oxidants can, however, be helpful; macrophages upon activation produce NO which kills pathogens (Marletta *et al.*, 1988; Nathan and Shiloh, 2000). NO is also used as a signalling molecule, its actions promoted through the intracellular messenger cyclic guanosine monophosphate (cGMP; Bellamy *et al.*, 2002; Fiscus, 2002). Oxidants are produced during normal cellular respiration, a product of the mitochondrial respiratory chain and these reactive molecules need to be detoxified before they cause damage to the cell (Lenaz *et al.*, 2002). They are usually classed as either reactive oxygen species (ROS) or reactive nitrogen species and can either be free radicals or non-radical reactive species. Free radicals are defined as having an unpaired electron in the outer orbit; in order to

complete their outer ring the radical species takes an electron from other molecules (Fang *et al.*, 2002), resulting in a chain reaction that can lead to lipid peroxidation and oxidation of DNA and proteins (Gutteridge and Smith, 1988). Non-radical species including hydrogen peroxide and peroxynitrite are formed when free radicals react with other compounds.

To control oxidant levels, cells have evolved a variety of mechanisms to deal with the stress associated with the production of ROS. These mechanisms include production of glutathione (GSH), the vitamins C (ascorbic acid) and E ( $\alpha$  tocopherol) and the enzymes superoxide dismutase and catalase. In most cells the main antioxidant defence is provided by the thiol-disulfide GSH formed from the amino acids glutamic acid, L-cysteine and glycine, assembled by the enzymes  $\gamma$ -glutamylcysteine synthetase and glutathione synthase (Johnston and Bloch, 1951; Sies *et al.*, 1999). GSH donates an electron detoxifying the radical while forming the glutathionyl radical, which then reacts with another glutathionyl radical resulting in the formation of glutathione disulphide (GSSG) which is then reduced back to GSH by glutathione reductase (Sies *et al.*, 1999). In most cells the ratio of GSH to GSSG is typically  $>30:1$  (Schafer and Buettner, 2001) and is used as a determining factor in measuring the oxidative stress of the cell. Levels of GSH in the cytosol of cells vary from 1-10 mM; although extra-cellular pools do exist they tend to be in the range of 1-10  $\mu$ M (Smith *et al.*, 1996) with levels in whole blood of humans around 2 mM (Ames *et al.*, 1981).

Ascorbic acid and  $\alpha$  tocopherol provide electrons to free radicals, however, they have little capacity to grab electrons from proteins, lipids or DNA themselves, thereby breaking the chain of events leading to oxidative damage (Sharma and Buettner, 1993; Winterbourn, 1993).

In human serum the main antioxidants are ascorbic acid and uric acid. Human serum levels of ascorbic acid vary from 20-90  $\mu$ M (Halliwell, 2001) while serum levels of uric acid are around 160-450  $\mu$ M (Ames *et al.*, 1981). About 60 million years ago the gene for ascorbic acid synthesis and uricase, the enzyme that breaks down uric acid were lost from the common ancestor of chimpanzees and humans. Both the synthesis of ascorbic

acid and breakdown of uric acid results in the production of hydrogen peroxide and it has been postulated that the loss of ascorbic acid synthesis and loss of uricase are linked (Ames *et al.*, 1981) Ascorbic acid is now required in the diet of humans and the concentration of uric acid in the blood has been increased by a factor of 10 compared to animals which possess a functional uricase (Ames *et al.*, 1981).

The enzymatic antioxidants superoxide dismutase (Noor *et al.* 2002) and catalase (Michiels *et al.*, 1994) are specific for superoxide and hydrogen peroxide respectively. Superoxide dismutase combines two superoxide molecules and two hydrogen ions to produce two molecules of hydrogen peroxide which can be broken down into two molecules of water by catalase.

#### **4.1.3.1 Antioxidants of trypanosomes**

The Kinetoplastida branched early in eukaryotic evolution and have many unique pathways especially those involved in reducing oxidative stress. Trypanosomes were thought to be highly susceptible to oxidative stress, due to the lack of glutathione reductase and catalase (Meshnick *et al.*, 1976; 1977). However, trypanosomes contain a barrage of low molecular weight antioxidants including trypanothione (Fairlamb *et al.*, 1985) and ovothiol A (Ariyanayagam and Fairlamb, 2001), the enzymes tryparedoxin (Lüdemann *et al.*, 1998) and glutathione peroxidases (Hillebrand *et al.*, 2003) presenting formidable cellular defences against oxidants.

The main antioxidant in trypanosomes is trypanothione, composed of two molecules of GSH linked by one molecule of spermidine (Fairlamb *et al.*, 1985). As much as 70% of the cell's GSH is transformed into trypanothione by the enzyme trypanothione synthetase (Fairlamb and Cerami, 1992) which is present in all Kinetoplastida including the insect pathogen *Crithidia* and the mammalian pathogens *Leishmania* spp. and *T. cruzi*. Oxidised trypanothione is reduced by trypanothione reductase, a system analogous to the glutathione-glutathione reductase system found in other eukaryotes. RNA



interference (RNAi) experiments have shown that the enzyme  $\gamma$ -glutamylcysteine synthetase is essential to trypanosomes (Huynh *et al.*, 2003).

The ovothiols were first characterised in the eggs of marine echinoderms and molluscs and are thought to play an important detoxifying role in response to the intense respiratory burst that accompanies fertilisation (Turner *et al.*, 1988a). Although found at concentrations over 1 mM in insect stages of *Leishmania*, the concentration of ovothiol A in *T. brucei* procyclics is around 10-fold less and is absent in bloodstream forms; it is thought that ovothiol A plays only a limited role in the scavenging of hydrogen peroxide as its concentration is far lower than trypanothione (Ariyanayagam and Fairlamb, 2001). *T. brucei* contains two thio-disulphide oxidoreductases, a tryparedoxin which is distantly related to the thioredoxin family (Lüdemann *et al.*, 1998) and a classical thioredoxin (Reckenfelderbaumer *et al.*, 2000). The thiol-disulphide oxidoreductases are found in all living cells and are characterised by the amino acid active site motif CXXC (cysteine, X, X, cysteine); they are involved in the reduction of ribonucleotide reductase which is important in DNA synthesis (Jordan and Reichard, 1998) and in reduction of glutathione peroxidases through a couple with trypanothione (Hillebrand *et al.*, 2003). The tryparedoxin active site contains a CPPC motif similar to the thioredoxins (CGPC), glutaredoxins (CPYC) and eukaryotic protein-disulfide isomerases (CGHC). *T. brucei* tryparedoxin shows characteristics of both the thioredoxins and glutaredoxins and it was suggested that it may form a new class of thiol-disulfide oxidoreductases (Lüdemann *et al.*, 1998). High levels of tryparedoxin are found within the trypanosome; however, the concentration of thioredoxin is three orders of magnitude lower than tryparedoxin (Schmidt and Krauth-Siegel, 2003) suggesting that thioredoxin does not play a significant role in parasite synthesis of DNA precursors. RNAi experiments to reduce levels of thioredoxin had no effect on culture forms of *T. brucei* (Schmidt *et al.*, 2002) indicating that tryparedoxin probably assumes the role of thioredoxin in this trypanosome (Schmidt and Krauth-Siegel, 2003).

Classical glutathione peroxidases are selenocysteines catalysing the reduction of hydroperoxides. They form a family consisting of four members (GPX1-4) and are found in different cellular locations. These include the cytosolic GPX 1, gastrointestinal



GPX 2, the GPX 3 found in human plasma and GPX 4 the phospholipid hydroperoxide glutathione peroxidase (Arthur, 2000). So far five distinct peroxidases have been found in the Trypanosomatidae. Two members of the peroxiredoxin family of antioxidant enzymes have been identified in *T. brucei* (Tetaud *et al.*, 2001) and *T. cruzi* (Wilkinson *et al.*, 2000). They are located in either the cytosol or mitochondrion and use trypanredoxin as an electron donor and reduce substrates, including hydrogen peroxide and small chain organic hydroperoxides to water and alcohols respectively (Alphey *et al.*, 2000). An ascorbic dependent peroxidase specific to hydrogen peroxide and related to the heme peroxidases found in plants has been identified in the endoplasmic reticulum of *T. cruzi* (Wilkinson *et al.*, 2002a). The two remaining proteins share extensive similarity to the non-selenium glutathione-dependent peroxidase family. GPXI has been found in both the cytosol and glycosomes of *T. cruzi* (Wilkinson *et al.*, 2002b) and the mitochondrion of *T. brucei* (Hillebrand *et al.*, 2003).

#### 4.1.3.2 Insect antioxidant systems

Compared to mammalian cells, which are exposed to oxygen concentrations of about 5%, insect cells are exposed to atmospheric concentrations of 20%. *Drosophila* uses GSH as its major cellular reducing agent, however, it lacks glutathione peroxidase (Smith and Shrift, 1978) and a typical glutathione reductase; instead GSSG is cleaved non-enzymatically by thioredoxin reductase (Kanzok *et al.*, 2001). The same is true in the mosquito *Anopheles gambiae* (Bauer *et al.*, 2003) and is therefore thought to be a common feature of the diptera, suggesting that tsetse will also lack glutathione reductase.

*Drosophila* lacking superoxide dismutase show reduced lifespans, males are sterile and females show reduced fecundity and are more susceptible to substances that increase oxidative stress (Phillips *et al.*, 1989). Catalase mutants show reduced lifespans but with no effect on fertility (Mackay and Bewley, 1989). *Drosophila* that cannot synthesise uric acid are more susceptible to oxidative damage caused by paraquat, ionizing radiation

and hyperoxia (Hilliker *et al.*, 1992). The major antioxidant in the haemolymph of *R. prolixus* has been shown to be uric acid which is present at concentrations up to 5 mM (Souza *et al.*, 1997). *R. prolixus* feeds on vertebrate blood and uric acid levels rise after blood feeding. During bloodmeal digestion large amounts of pro-oxidants are produced in *R. prolixus* including hemin and iron which drive Fenton reactions, producing hydroxyl ions which are highly oxidative (Souza *et al.*, 1997). Uric acid is the main end product of nitrogen metabolism in insects and makes up over 70% of tsetse excretions (Bursell, 1965). Levels of uric acid in tsetse pupae rise rapidly during the first 5 d of pupation from 0.09  $\mu$ M to a steady concentration of 0.37  $\mu$ M, then fall quickly after emergence to concentrations of 0.06  $\mu$ M (Brown *et al.*, 1973). In response to a bloodmeal the activity of xanthine oxidase ( $\text{Xanthine} + \text{O}_2 + 2\text{H}_2\text{O} \rightarrow \text{Uric acid} + 2\text{H}_2\text{O}_2$ ) rises (measured as nM of NADH formed per insect) from 1.01 to 6.08 nM after 24 h and 3 d respectively, then begins to fall (Brown *et al.*, 1973).

#### **4.1.4 Cellular signalling**

##### **4.1.4.1 cAMP**

Cyclic adenosine monophosphate (cAMP) was first shown to be involved in signal transmission by Rall *et al.* (1957). Since then it has been found to act as a second messenger in all organisms including bacteria, fungi, mammals and higher plants (Richards *et al.*, 2002). cAMP is produced from adenosine triphosphate (ATP) by the enzyme adenylate cyclase and is degraded to inactive AMP by phosphodiesterases (Francis *et al.*, 2001). cAMP exerts the majority of its functions by activating protein kinase A, resulting in differential gene expression due to the phosphorylation of transcription factors (Frisch, 2000).

It has been estimated that trypanosomes contain more than 100 genes for different adenylate cyclases; in comparison mammals have only 10 adenylate cyclases (Sunahara *et al.*, 1996; Seebeck *et al.*, 2001). Trypanosomal adenylate cyclases generally have

conserved catalytic domains; however, the N-terminal (extracellular) domains vary greatly. They are structurally similar to the transmembrane guanylate cyclases of multicellular organisms and it has been hypothesised that the adenylate cyclases found in trypanosomes may serve as extracellular receptors (Seebeck *et al.*, 2001).

cAMP has been implicated in the differentiation of trypanosomes in the bloodstream (Vassella *et al.*, 1997) and from bloodstream to procyclic forms. Transformation from long slender to short stumpy forms has been shown to be density dependent and is under the control of stumpy induction factor (SIF) an effect mimicked by cAMP analogues. During *in vitro* differentiation of bloodstream to procyclic form, two peaks of adenylate cyclase activity have been observed during the *in vitro* transformation of a monomorphic strain of *T. brucei* (Rolin *et al.*, 1993). The first peak occurs 6-10 h, immediately after the release of the VSG coat and before the first cell division; the second peak occurs when the cells begin to proliferate after the trypanosomes have emerged from the first division cycle.

Two families of cAMP phosphodiesterases have been found in *T. brucei*. TbPDE1 is the only representative of family 1 and has been shown not to be essential in culture or during midgut infection of tsetse flies (Gong *et al.*, 2001). The second family contains three different phosphodiesterases TbPDE2A (Zoraghi *et al.*, 2001), TbPDE2B (Rascon *et al.*, 2002), TbPDE2C (Zoraghi and Seebeck, 2002) which have been shown to be essential in *T. brucei*. RNA interference of TbPDE2C leads to the disruption of nuclear and cellular division and to cell death (Zoraghi and Seebeck, 2002).

#### **4.1.4.2 cGMP**

Although cGMP was discovered 40 years ago (Ashman *et al.*, 1963), knowledge of its cellular functions has lagged behind that of cAMP which is mainly due to it being present at 10 to 50-fold lower concentrations than cAMP. Guanylate cyclases convert guanosine triphosphate (GTP) into cGMP which is then degraded to GMP by phosphodiesterases (Francis *et al.*, 2001). Two different types of guanylate cyclases

exist in the cell: soluble and transmembrane. cGMP is synthesised in response to a variety of signals; these include NO, peptide ligands and fluxes in intracellular concentrations of calcium ions. Soluble guanylate cyclases are expressed in the cytoplasm of almost all mammalian cells (Lucas *et al.*, 2000) and mediate a wide range of important physiological functions including inhibition of platelet aggregation, relaxation of smooth muscle, vasodilatation, neuronal signal transduction and immunomodulation (Collier and Vallance, 1989). Soluble guanylate cyclases are activated by NO producing cGMP (Arnold *et al.*, 1977). Transmembrane guanylate cyclases span the cell membrane and are classified as either natriuretic peptide receptors, intestinal peptide-binding receptors or orphan receptors (Lucas *et al.*, 2000). Binding of the ligand activates transmembrane guanylate cyclase, resulting in an increase in cGMP concentration within the cell (Wong and Garbers, 1992). Increasing cellular concentrations of cGMP leads to activation of protein kinases, changes in ion channels and changes in levels of cyclic nucleotides by regulation of phosphodiesterases (Lucas *et al.*, 2000).

Investigations into signalling mechanisms involving cGMP have increased in recent years as NO is involved in activating the soluble form of guanylate cyclase. So far little work has been done on cGMP signalling in trypanosomes although recently a cGMP dependent enzyme activity has been discovered in *Leishmania* (Geigel and Leon, 2003) and a protein kinase A has been shown to function through cGMP in *T. brucei* (Shalaby *et al.*, 2001). A NO signal transduction pathway has been found in *T. cruzi* (Paveto *et al.*, 1995; Pereira *et al.*, 1997) which suggests that cGMP could have an important signalling function in the Kinetoplastida.

#### **4.1.5 The present work**

In the present work, to examine if changing the oxidative environment in the fly midgut could influence trypanosome infection rates, a range of antioxidants were added to the infective bloodmeal. Apoptotic pathways can be influenced by cyclic nucleotides and

trypanosome differentiation of bloodstream forms has been shown to be under the control of SIF operating through cAMP. There has, however, been little work to investigate whether cyclic nucleotides play a role in the differentiation of bloodstream to procyclic forms, so the effects of the cyclic nucleotides, cAMP and cGMP on midgut infection rates are also investigated.

## 4.2 Results

See Section 2.16 for details on trypanosome production, Section 2.17 for details on infection and dissection of tsetse flies and Section 2.26.7 for details on statistical analysis. Unless otherwise stated see Section 2.18 for details on compounds that were fed to tsetse flies.

### 4.2.1 The effects of GSH or GSSG on midgut infection rates of *T. b. brucei* in *G. m. morsitans*

The effects on midgut infection rates of *T. b. brucei* BUT 135 in *G. m. morsitans* when the bloodmeal was supplemented with GSH are shown in Figure 4.1.

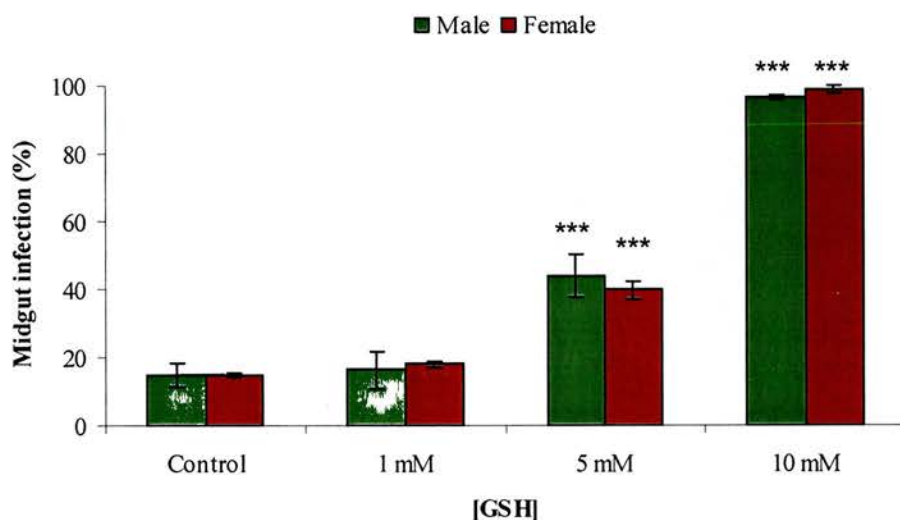


Figure 4.1: Effects of GSH on midgut infection rates of *T. b. brucei* in *G. m. morsitans*. Data presented as the mean  $\pm$  Standard error of mean (S.E.M) from three experiments.  $\chi^2$  tests: \*\*\*  $p < 0.001$  versus the corresponding control value.

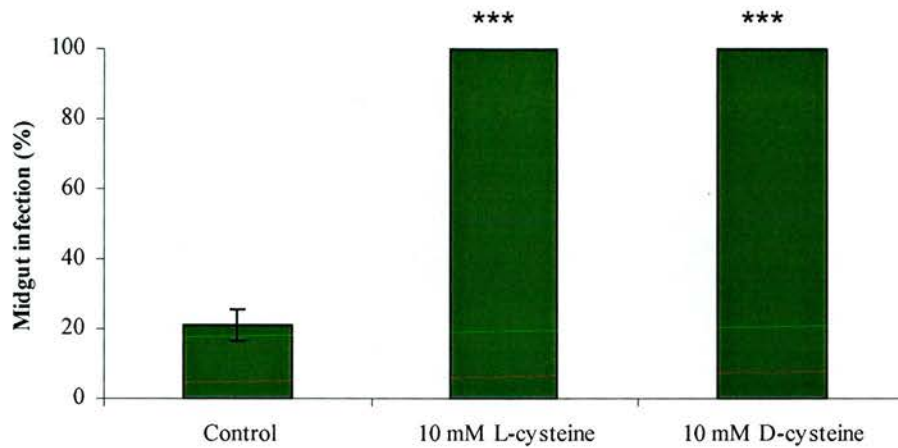
Addition of 5 mM or 10 mM GSH significantly increased midgut infection rates of BUT 135 from a control value of 15% (n=88) in male *G. m. morsitans* to 44% (n=91) and 97% (n=92) respectively and from a control value of 15% (n=88) in female *G. m. morsitans* to 40% (n=93) and 99% (n=93) respectively (male: 5 mM:  $\chi^2=18.3$ ,  $p<0.001$ ; 10 mM:  $\chi^2=123.1$ ,  $p<0.001$ ; female: 5 mM:  $\chi^2=14.1$ ,  $p<0.001$ ; 10 mM:  $\chi^2=131.5$ ,  $p<0.001$ ). There was no significant difference between control flies and those given 1 mM GSH in either males or females which resulted in infections rates of 16% (n=92) and 18% (n=83) respectively (male:  $\chi^2<0.1$ ,  $p=0.777$ ; female:  $\chi^2=0.3$ ,  $p=0.560$ ). The differences between 1 mM or 5 mM GSH, 1 mM or 10 mM GSH and 5 mM or 10 mM GSH were also significant in both male and female flies (male: 1 mM compared to 5 mM:  $\chi^2=16.6$ ,  $p<0.001$ ; 1 mM compared to 10 mM:  $\chi^2=121.1$ ,  $p<0.001$ ; 5 mM compared to 10 mM:  $\chi^2=61.3$ ,  $p<0.001$ ; female: 1 mM compared to 5 mM:  $\chi^2=9.93$ ,  $p<0.001$ ; 1 mM compared to 10 mM:  $\chi^2=120.3$ ,  $p<0.001$ ; 5 mM compared to 10 mM:  $\chi^2=76.5$ ,  $p<0.001$ ). There were no significant differences between midgut infection rates of control male or control female flies or those fed either of the doses of GSH (control:  $\chi^2<0.11$ ,  $p=0.860$ ; 1 mM:  $\chi^2<0.1$ ,  $p=0.757$ ; 5 mM:  $\chi^2<0.1$ ,  $p=0.566$ ; 10 mM:  $\chi^2=1.044$ ,  $p=0.307$ ).

The addition of 15 mM GSSG significantly increased midgut infection rates in male tsetse from a control value of 13% (n=72) to 98% (n=59;  $\chi^2=95.5$ ,  $p<0.001$ ).

#### **4.2.2 The effects of cysteine on midgut infection rates of *T. b. brucei* in *G. m. morsitans***

The effects on midgut infection rates of *T. b. brucei* BUT 135 in *G. m. morsitans* when the bloodmeal was supplemented with 10 mM L- or D-cysteine are shown in Figure 4.2.





**Figure 4.2: Effects of 10 mM cysteine on midgut infection rates of *T. b. brucei* in *G. m. morsitans*.** Data presented as the mean  $\pm$  S.E.M from three experiments.  $\chi^2$  tests: \*\*\*  $p < 0.001$  versus the corresponding control value.

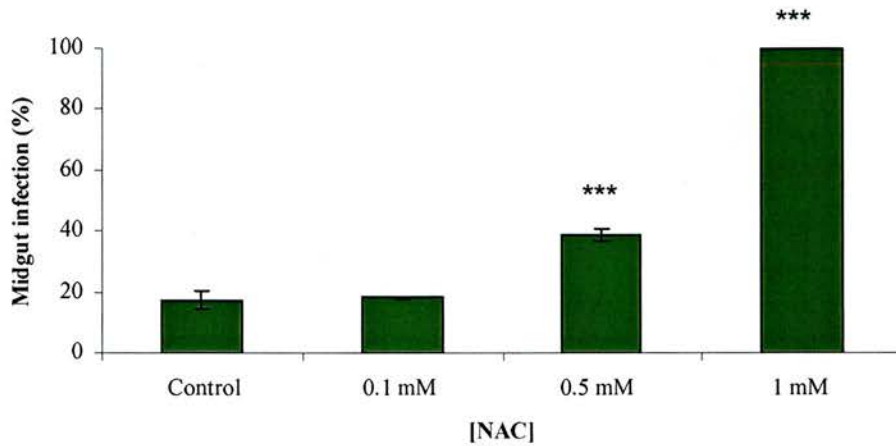
The addition of either 10 mM L- or D-cysteine significantly increased infection rates from a control value of 21% (n=119) to 100% (L-cysteine: n=117:  $\chi^2=153.6$ ,  $p < 0.001$ ; D-cysteine: n=113:  $\chi^2=150.1$ ,  $p < 0.001$ ).

The addition of 5 mM L or D-cysteine significantly increased midgut infection rates from a control value of 10% (n=29) to 37% (n=30) and 36% (n=28) respectively (L-cysteine:  $\chi^2=5.6$ ,  $p=0.018$ ; D-cysteine  $\chi^2=5.2$ ,  $p=0.022$ ). The addition of 1 mM L- or D-cysteine had no significant effect on midgut infection rates which were 12% (n=33) in control flies compared to 16% (n=31) and 12% (n=32) in those fed L- or D-cysteine respectively (L-cysteine:  $\chi^2=0.2$ ,  $p=0.645$ ; D-cysteine  $\chi^2 < 0.1$ ,  $p=0.964$ ).



### 4.2.3 The effects of NAC on midgut infection rates of *T. b. brucei* in *G. m. morsitans*

The effects on midgut infection rates of *T. b. brucei* BUT 135 in *G. m. morsitans* when the bloodmeal was supplemented with NAC are shown in Figure 4.3.

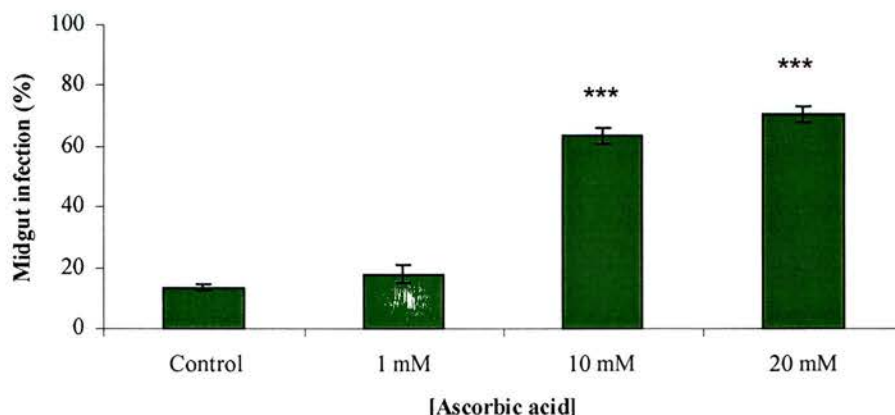


**Figure 4.3:** Effects of NAC on midgut infection rates of *T. b. brucei* in *G. m. morsitans*. Data presented as the mean  $\pm$  S.E.M from three experiments.  $\chi^2$  tests: \*\*\*  $p < 0.001$  versus the corresponding control value.

Addition of 0.5 mM or 1 mM NAC significantly increased midgut infection rates of BUT 135 in male *G. m. morsitans* from a control value of 17% (n=103) to 39% (n=98) and 100% (n=106) respectively (0.5 mM:  $\chi^2=11.3$ ,  $p < 0.001$ ; 1 mM:  $\chi^2=147.4$ ,  $p < 0.001$ ). Addition of 0.1 mM NAC resulted in midgut infection rates of 18% (n=98) which were not significantly different from the controls ( $\chi^2=0.1$ ,  $p=0.869$ ). The differences between the addition of 0.1 or 0.5 mM, 0.1 mM or 1 mM and 0.5 mM or 1 mM NAC were also significant (0.1 mM compared to 0.5 mM:  $\chi^2=10.0$ ,  $p=0.002$ ; 0.1 mM compared to 1 mM: ( $\chi^2=142.4$ ,  $p < 0.001$ ; 0.5 mM compared to 1 mM:  $\chi^2=91.9$ ,  $p < 0.001$ ).

#### 4.2.4 The effects of ascorbic acid on midgut infection rates of *T. b. brucei* in *G. m. morsitans*

The effects on midgut infection rates of *T. b. brucei* BUT 135 in *G. m. morsitans* when the bloodmeal was supplemented with ascorbic acid are shown in Figure 4.4.



**Figure 4.4:** Effects of ascorbic acid on midgut infection rates of *T. b. brucei* in *G. m. morsitans*. Data presented as the mean  $\pm$  S.E.M from three experiments.  $\chi^2$  tests: \*\*\*  $p < 0.001$  versus the corresponding control value.

The addition of 10 or 20 mM ascorbic acid significantly increased midgut infection rates of BUT 135 in male *G. m. morsitans* from a control value of 14% (n=95) to 64% (n=94) and 71% (n=89) respectively (10 mM:  $\chi^2=50.1$ ,  $p < 0.001$ ; 20 mM:  $\chi^2=61.8$ ,  $p < 0.001$ ). Addition of 1 mM ascorbic acid resulted in midgut infection rates of 18% (n=100) which was not significantly different from the control value ( $\chi^2=0.7$ ,  $p=0.410$ ). The differences between the addition of 1 mM or 10 mM ascorbic acid and 1 mM or 20 mM ascorbic acid were also significant (1 mM compared to 10 mM:  $\chi^2=42.3$ ,  $p < 0.001$ ; 1 mM compared to 20 mM:  $\chi^2=53.6$ ,  $p < 0.001$ ). There was no significant difference between the addition of 10 mM or 20 mM ascorbic acid ( $\chi^2=1.0$ ,  $p=0.316$ ). Increasing the dose of ascorbic acid to 30 mM had a toxic effect on the tsetse (data not shown).

#### 4.2.5 The effect of uric acid on midgut infection rates of *T. b. brucei* in *G. m. morsitans*

The effects on midgut infection rates of *T. b. brucei* BUT 135 in *G. m. morsitans* when the bloodmeal was supplemented with uric acid are shown in Figure 4.5.

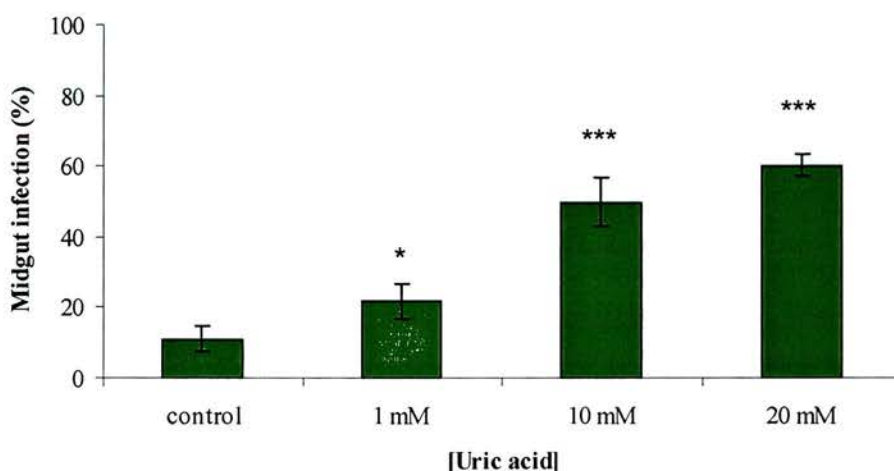


Figure 4.5: Effects of uric acid on midgut infection rates of *T. b. brucei* in *G. m. morsitans*. Data presented as the mean  $\pm$  S.E.M from three experiments.  $\chi^2$  tests: \*\*\*  $p < 0.001$ ; \*  $p < 0.05$  versus the corresponding control value.

The addition of 1 mM, 10 mM or 20 mM uric acid significantly increased midgut infection rates of BUT 135 in male *G. m. morsitans* from a control value of 11% (n=99) to 22% (n=96), 50% (n=87) and 60% (n=83) respectively (1 mM:  $\chi^2=4.4$ ,  $p=0.036$ ; 10 mM:  $\chi^2=34.6$ ,  $p<0.001$ ; 20 mM:  $\chi^2=48.9$ ,  $p<0.001$ ). Significant differences were also found between the addition of 1 mM or 10 mM uric acid and 1 mM or 20 mM uric acid (1 mM compared to 10 mM:  $\chi^2=15.6$ ,  $p<0.001$ ; 1 mM compared to 20 mM:  $\chi^2=26.4$ ,  $p<0.001$ ). There were no significant differences between the addition of 10 mM or 20 mM uric acid ( $\chi^2=1.6$ ,  $p=0.205$ ). The addition of 10 mM or 20 mM uric acid significantly increased midgut infection rates in female *G. m. morsitans* from a control value of 14% (n=37) to 57% (n=36) and 61% (n=36) respectively (10 mM:  $\chi^2=15.1$ ,

$p<0.001$ ; 20 mM:  $\chi^2=17.7$ ,  $p<0.001$ ). The addition of 1 mM uric acid raised midgut infection rates to 23%, however, this was not significant ( $\chi^2=1.0$ ,  $p=0.329$ ). There were no significant differences in midgut infection rates between male and female *G. m. morsitans* with the addition of uric acid (control:  $\chi^2=0.1$ ,  $p=0.699$ ; 1 mM:  $\chi^2<0.1$ ,  $p=0.974$ ; 10 mM:  $\chi^2=0.4$ ,  $p=0.511$ ; 20 mM:  $\chi^2<0.1$ ,  $p=0.928$ ). Increasing the uric acid concentration of the infective feed to 60 mM had an antifeedant effect and no flies had fed after 1 h (data not shown).

#### 4.2.6 The effects of 8-Br-cGMP on midgut infection rates of *T. b. brucei* in *G. m. morsitans*

The effects on midgut infection rates of *T. b. brucei* BUT 135 in *G. m. morsitans* when the bloodmeal was supplemented with 8-Br-cGMP are shown in Figure 4.6

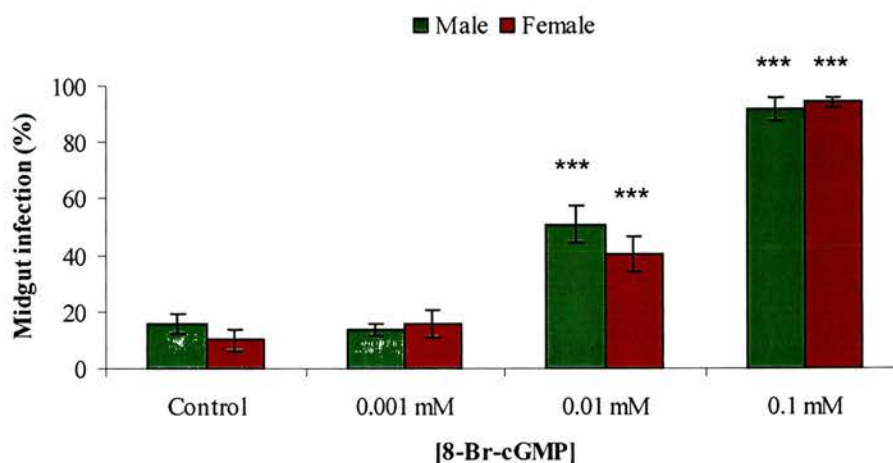


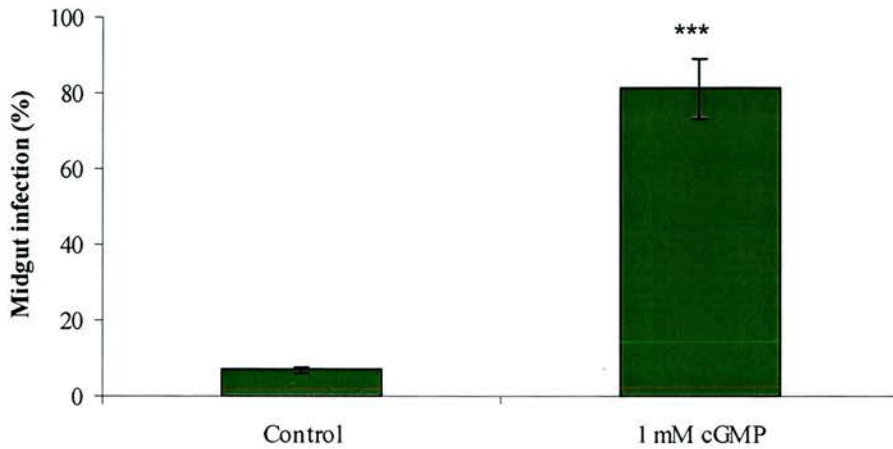
Figure 4.6: Effects of 8-Br-cGMP on midgut infection rates of *T. b. brucei* in *G. m. morsitans*. Data presented as the mean  $\pm$  S.E.M from three experiments.  $\chi^2$  tests: \*\*\*  $p<0.001$  versus the corresponding control value.

Addition of 10  $\mu$ M or 100  $\mu$ M 8-Br-cGMP significantly increased midgut infection rates of BUT 135 from a control value of 16% (n=95) in male *G. m. morsitans* to 51% (n=106) and 92% (n=97) respectively and from a control value of 10% (n=98) in female *G. m. morsitans* to 40% (n=109) and 94% (n=105) respectively (male: 10  $\mu$ M:  $\chi^2=27.5$ ,  $p<0.001$ ; 100  $\mu$ M:  $\chi^2=104.6$ ,  $p<0.001$ ; female: 10  $\mu$ M:  $\chi^2=24.3$ ,  $p<0.001$ ; 100  $\mu$ M:  $\chi^2=144.1$ ,  $p<0.001$ ). There were no significant differences between the control and addition of 1  $\mu$ M 8-Br-cGMP in male or female flies which resulted in infections rates of 13% (n=86) and 16% (n=94) respectively (male:  $\chi^2=0.2$ ,  $p=0.639$ ; female:  $\chi^2=1.4$ ,  $p=0.236$ ). The differences between the addition of 1 or 10  $\mu$ M 8-Br-cGMP, 1 or 100  $\mu$ M 8-Br-cGMP and 10 or 100  $\mu$ M 8-Br-cGMP were also significant in both male and female flies (male: 1  $\mu$ M compared to 10  $\mu$ M:  $\chi^2=32.3$ ,  $p<0.001$ ; 1  $\mu$ M compared to 100  $\mu$ M:  $\chi^2=112.2$ ,  $p<0.001$ ; 10  $\mu$ M compared to 100  $\mu$ M:  $\chi^2=37.3$ ,  $p<0.001$ ; female: 1  $\mu$ M compared to 10  $\mu$ M:  $\chi^2=14.6$ ,  $p<0.001$ ; 1  $\mu$ M compared to 100  $\mu$ M:  $\chi^2=124.4$ ,  $p<0.001$ ; 10  $\mu$ M compared to 100  $\mu$ M:  $\chi^2=70.1$ ,  $p<0.001$ ). There were no significant differences between control male or control females or those fed either of the doses of 8-Br-cGMP (control:  $\chi^2=1.3$ ,  $p=0.248$ ; 1  $\mu$ M:  $\chi^2=0.2$ ,  $p=0.618$ ; 10  $\mu$ M:  $\chi^2=2.4$ ,  $p=0.120$ ; 100  $\mu$ M:  $\chi^2=0.4$ ,  $p=0.508$ ).

#### **4.2.7 The effects of cGMP on midgut infection rates of *T. b. brucei* in *G. m. morsitans***

The effects on midgut infection rates of *T. b. brucei* BUT 135 in *G. m. morsitans* when the bloodmeal was supplemented with cGMP are shown in Figure 4.7.





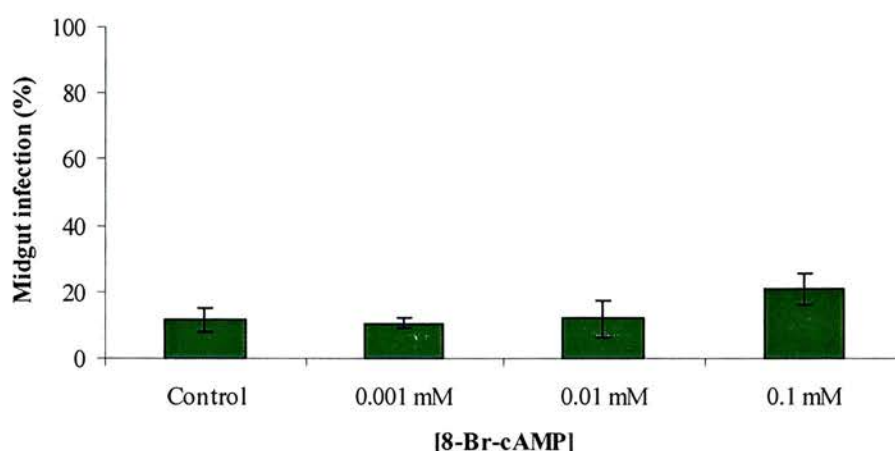
**Figure 4.7: Effects of cGMP on midgut infection rates of *T. b. brucei* in *G. m. morsitans*. Data presented as the mean  $\pm$  S.E.M from three experiments.  $\chi^2$  tests: \*\*\*  $p < 0.001$  versus the corresponding control value.**

Addition of 1 mM cGMP significantly increased midgut infection rates of BUT 135 in male *G. m. morsitans* from a control value of 7% (n=72) to 81% (n=81;  $\chi^2=85.1$ ,  $p < 0.001$ ).

The addition of 1 mM cGMP significantly increased midgut infection rates of GUT 3.1 in male *G. m. morsitans* from a control value of 37% (n=41) to 90% (n=29) and from 34% (n=65) to 69% (n=67) in female *G. m. morsitans* (male:  $\chi^2=19.7$ ,  $p < 0.001$ ; female:  $\chi^2=16.0$ ,  $p < 0.001$ ). Addition of 1 mM cGMP significantly increased midgut infection rates of GUT 3.1 in male *G. m. centralis* from a control value of 23% (n=75) to 74% (n=66) and from 42% (n=65) to 90% (n=60) in female *G. m. centralis* (male:  $\chi^2=37.5$ ,  $p < 0.001$ ; female:  $\chi^2=32.1$ ,  $p < 0.001$ ).

#### 4.2.8 The effects of 8-Br-cAMP on midgut infection rates of *T. b. brucei* in *G. m. morsitans*

The effects on midgut infection rates of *T. b. brucei* BUT 135 in *G. m. morsitans* when the bloodmeal was supplemented with 8-Br-cAMP are shown in Figure 4.8.



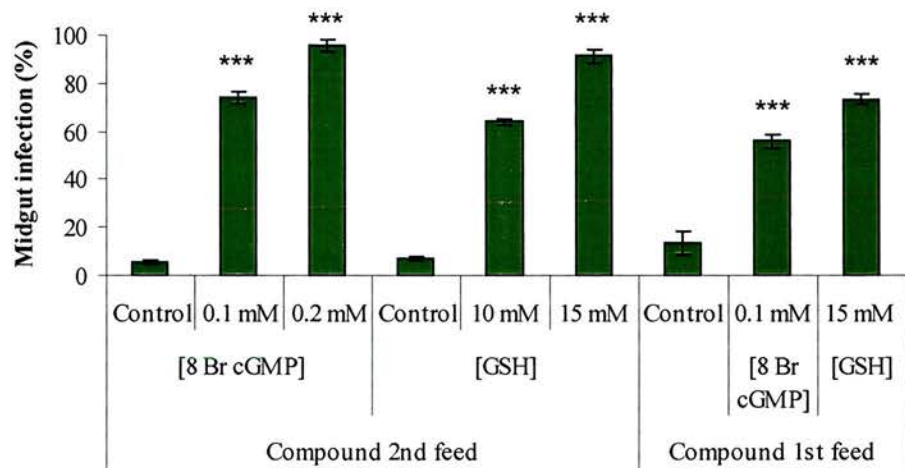
**Figure 4.8:** Effects of 8-Br-cAMP on midgut infection rates of *T. b. brucei* in *G. m. morsitans*. Data presented as the mean  $\pm$  S.E.M from three experiments.

The addition of 1  $\mu$ M, 10  $\mu$ M or 100  $\mu$ M 8-Br-cAMP had no significant effect on midgut infection rates in male *G. m. morsitans* when compared to controls. Midgut infection rates were 12% (n=119), 11% (n=111), 12% (n=116) and 21% (n=120) for control flies and those fed 1  $\mu$ M, 10  $\mu$ M or 100  $\mu$ M 8-Br-cAMP respectively (1  $\mu$ M:  $\chi^2 < 0.1$ ,  $p = 0.820$ ; 10  $\mu$ M:  $\chi^2 < 0.1$ ,  $p = 0.944$ ; 100  $\mu$ M  $\chi^2 = 3.6$ ,  $p = 0.058$ ). There were no significant differences between flies fed 10  $\mu$ M 8-Br-cAMP or flies fed 1  $\mu$ M or 100  $\mu$ M 8-Br-cAMP (1  $\mu$ M:  $\chi^2 < 0.1$ ,  $p = 0.765$ ; 100  $\mu$ M:  $\chi^2 = 3.3$ ,  $p = 0.070$ ). The difference between the addition of 1  $\mu$ M or 100  $\mu$ M 8-Br-cAMP was significant ( $\chi^2 = 4.3$ ,  $p = 0.038$ ).

#### 4.2.9 The effects of 8-Br-cGMP, GSH, uric acid or ascorbic acid on midgut infection rates of *T. b. brucei* in non-teneral *G. m. morsitans*

See Sections 2.18.3 and 2.18.3 for methods.

The effects on midgut infection rates in non teneral *G. m. morsitans* of *T. b. brucei* BUT 135 when the bloodmeal was supplemented with 8-Br-cGMP or GSH in non-teneral flies or when teneral flies were fed 8-Br-cGMP or GSH and infected on the second feed are shown in Figure 4.9.



**Figure 4.9: Effects of 8-Br-cGMP or GSH on midgut infection rates of *T. b. brucei* on non teneral *G. m. morsitans*. Data presented as the mean  $\pm$  S.E.M from three experiments.  $\chi^2$  tests: \*\*\*  $p < 0.001$  versus the corresponding control value.**

The addition of 100  $\mu$ M or 200  $\mu$ M 8-Br-cGMP to the infective bloodmeal significantly increased midgut infection rates of BUT 135 in male *G. m. morsitans* from a control value of 6% ( $n=160$ ) to 74% ( $n=101$ ) and 96% ( $n=100$ ) respectively (100  $\mu$ M:  $\chi^2=133.6$ ,  $p < 0.001$ ; 200  $\mu$ M:  $\chi^2=208.8$ ,  $p < 0.001$ ). The difference between the addition of 100 or 200  $\mu$ M 8-Br-cGMP was also significant ( $\chi^2=18.7$ ,  $p < 0.001$ ). The addition of 10 or 15



mM GSH significantly increased midgut infection rates from a control value of 7% (n=136) to 64% (n=95) and 92% (n=108) respectively (10 mM:  $\chi^2=84.9$ ,  $p<0.001$ ; 15 mM:  $\chi^2=173.1$ ,  $p<0.001$ ). The difference between the addition of 10 or 15 mM GSH was also significant ( $\chi^2=22.8$ ,  $p<0.001$ ).

The addition of 20 mM ascorbic acid to the bloodmeal of non teneral flies significantly increased midgut infection rates from a control value of 6% (n=100) to 21% (n=109;  $\chi^2=10.0$ ,  $p=0.002$ ).

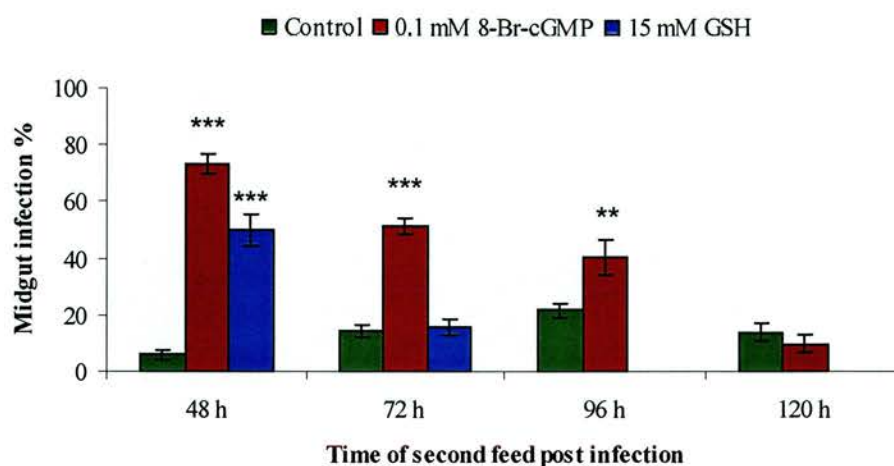
There was no significant difference between control flies and those fed 20 mM uric acid, producing midgut infection rates of 6% (n=100) and 3% (n=103) respectively ( $\chi^2=1.1$ ,  $p=0.285$ ).

The addition of 100  $\mu$ M 8-Br-cGMP or 15 mM GSH to the first bloodmeal with infection following on the second bloodmeal significantly increased midgut infection rates from a control value of 14% (n=95) to 56% (n=71) and 74% (n=87) respectively (8-Br-cGMP:  $\chi^2=34.0$ ,  $p<0.001$ ; GSH:  $\chi^2=66.7$ ,  $p<0.001$ ).

#### **4.2.10 The effects of 8-Br-cGMP, GSH or NAC when fed at different time points after infection on midgut infection rates of *T. b. brucei* in *G. m. morsitans***

See Section 2.18.4 for methods.

The effects on midgut infection rates in *G. m. morsitans* of *T. b. brucei* BUT 135 when the bloodmeal was supplemented with 8-Br-cGMP or GSH post-infection are shown in Figure 4.10



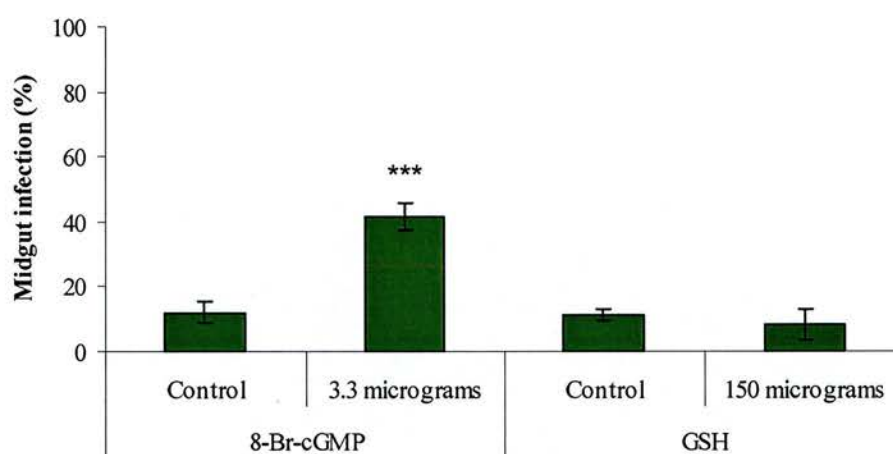
**Figure 4.10:** Effects of 8-Br-cGMP or GSH on midgut infection rates of *T. b. brucei* in *G. m. morsitans* when fed at different time points after infection. Data presented as the mean  $\pm$  S.E.M from three experiments.  $\chi^2$  tests: \*\*\*  $p < 0.001$ ; \*\*  $p < 0.01$  versus the corresponding control values.

The addition of 100  $\mu$ M 8-Br-cGMP to the bloodmeal at the second feed 48, 72 or 96 h post-infection with BUT 135 significantly increased midgut infection rates of male *G. m. morsitans* from control values of 6% (n=101), 14% (n=99) and 22% (n=97) to 73% (n=105), 52% (n=99) and 40% (n=99) respectively (48 h:  $\chi^2=97.2$ ,  $p < 0.001$ ; 72 h:  $\chi^2=31.4$ ,  $p < 0.001$ ; 96 h:  $\chi^2=8.0$ ,  $p=0.005$ ). The addition of 100  $\mu$ M 8-Br-cGMP 120 h post-infection produced midgut infection rates of 10% (n=83) which was not significantly different to the control value of 14% (n=79;  $\chi^2=0.7$ ,  $p=0.397$ ). The addition of 15 mM GSH 48 h post-infection significantly increased midgut infection rates from a control value of 6% (n=106) to 50% (n=101;  $\chi^2=49.3$ ,  $p < 0.001$ ). The addition of 15 mM GSH 72 h post-infection produced midgut infection rates of 16% (n=102) which was not significantly different to the control value of 14% ( $\chi^2=0.1$ ,  $p=0.759$ ). The addition of 5 mM NAC to the bloodmeal 72 h post-infection produced midgut infection rates of 10% (n=96) which was not significantly different to the control value of 9% (n=104;  $\chi^2=0.2$ ,  $p=0.671$ ).

#### 4.2.11 The effects of 8-Br-cGMP, GSH or NAC when injected into the haemolymph before infection on midgut infection rates of *T. b. brucei* in *G. m. morsitans*

See Section 2.18.5 for methods

The effects on midgut infection rates in *G. m. morsitans* of *T. b. brucei* BUT 135 when the tsetse were injected with either 3.3 µg 8-Br-cGMP or 150 µg GSH 6 h before infection are shown in Figure 4.11.



**Figure 4.11:** Effects of injecting 8-Br-cGMP or GSH into the haemolymph of *G. m. morsitans* on midgut infection rates of *T. b. brucei*. Data presented as the mean  $\pm$  S.E.M from three experiments.  $\chi^2$  tests: \*\*\*  $p < 0.001$  versus the corresponding control values.

Injection of 3.3 µg 8br cGMP into the haemolymph of *G. m. morsitans*, the equivalent of a bloodmeal dose of 200 µM significantly increased midgut infection rates from a control value of 12% (n=148) to 42% (n=118;  $\chi^2=30.0$ ,  $p < 0.001$ ).

Injection of with 150 µg of GSH into the haemolymph of *G. m. morsitans*, the equivalent of a bloodmeal dose of 15 mM had no effect on infection rates which were 11% (n=71) and 8% (n=74) for control flies and those injected with GSH respectively ( $\chi^2 < 0.1$ ,  $p = 0.803$ ).

Injection 5.4 µg NAC into the haemolymph of *G. m. morsitans*, the equivalent of a bloodmeal dose of 1 mM had no significant effect on infection rates which were 17% (n=35) and 15% (n=34) for control flies and those injected with NAC respectively ( $\chi^2 < 0.1$ ,  $p = 0.783$ ).

#### 4.2.12 The effects of NAC or 8-Br-cGMP on midgut infection rates of *T. b. brucei* when *G. m. morsitans* were injected with *E. coli* or *S. glossinidius* 24 h before infection with trypanosomes

See Sections 2.18.6 and 2.18.7 for methods.

The effects on midgut infection rates in *G. m. morsitans* of *T. b. brucei* BUT 135 when the flies were injected with *E. coli* 24 h before receiving the infective bloodmeal supplemented with NAC or 8-Br-cGMP are shown in Figure 4.12.

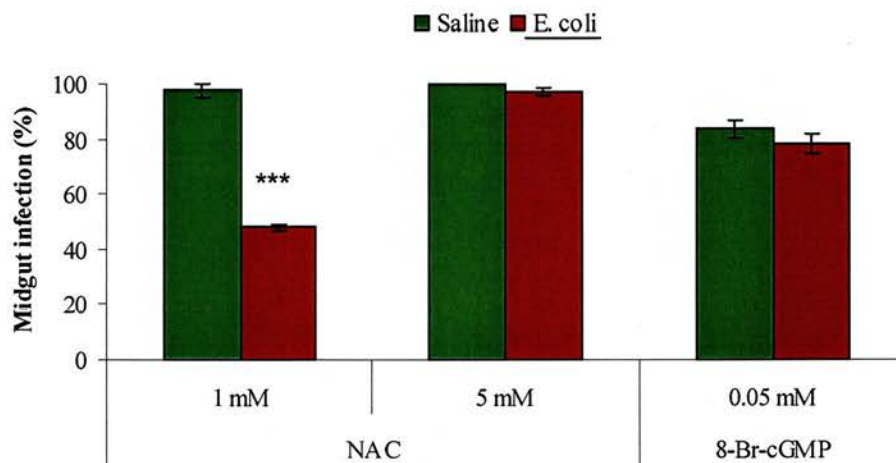


Figure 4.12: Effects of NAC or 8-Br cGMP on midgut infection rates when *G. m. morsitans* were injected with *E. coli* 24 h before infection with *T. b. brucei*. Data presented as the mean  $\pm$  S.E.M from three experiments.  $\chi^2$  tests: \*\*\*  $p < 0.001$  versus the corresponding saline control value.

Injection of *E. coli* into the haemolymph of *G. m. morsitans* 24 h before allowing the tsetse to feed on an infective bloodmeal containing 1 mM NAC significantly reduced susceptibility to trypanosome infection. Midgut infection rates fell from a control value of 98% (n=93) to 48% (n=75;  $\chi^2=55.9$ ,  $p<0.001$ ). The addition of 5 mM NAC to the infective feed had no significant effect on midgut infection rates which were 100% (n=91) in control flies compared to 97% (n=71) in those injected with *E. coli* ( $\chi^2=2.3$ ,  $p=0.107$ ).

The addition of 50  $\mu$ M 8-Br-cGMP to the infective feed resulted in no significant differences in midgut infection rates between control flies and those injected with *E. coli*. Midgut infection rates were 84% (n=116) in control flies compared to 78% (n=101) in those injected with *E. coli* ( $\chi^2=1.5$ ,  $p=0.227$ ).

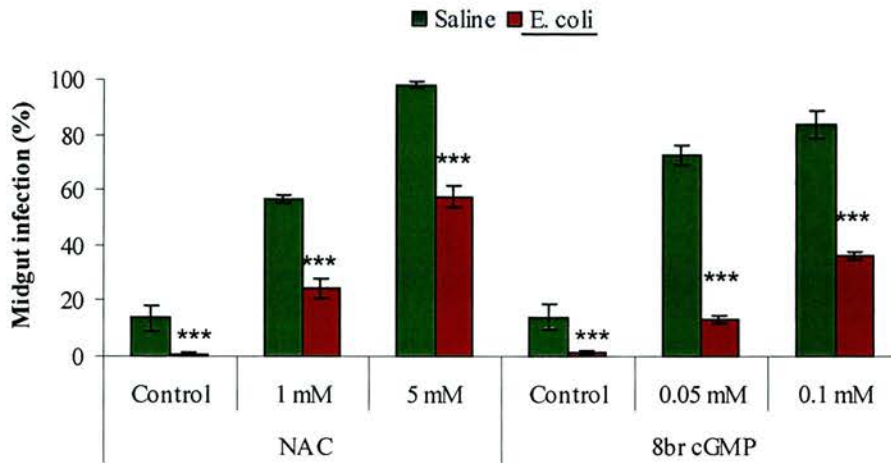
No significant differences were found between control tsetse and those injected with *S. glossinidius* isolated from either *G. m. morsitans* or *G. p. palpalis* when the infective bloodmeal was supplemented with 0.8 mM NAC. Midgut infection rates were 79% (n=28) in control flies compared to 84% (n=25) and 74% (n=27) in those injected with *S. glossinidius* from *G. m. morsitans* and *G. p. palpalis* respectively (*G. m. morsitans*:  $\chi^2=0.3$ ,  $p=0.614$ ; *G. p. palpalis*:  $\chi^2=0.2$ ,  $p=0.695$ ).

#### **4.2.13 The effects of NAC or 8-Br-cGMP on midgut infection rates of *T. b. brucei* when *G. m. morsitans* were fed *E. coli* 3 d before infection with *T. b. brucei***

See Section 2.18.8 for methods.

The effects on midgut infection rates in *G. m. morsitans* of *T. b. brucei* BUT 135 when the teneral bloodmeal was supplemented with *E. coli* and flies were infected with either NAC or 8-Br-cGMP at the second feed are shown in Figure 4.13





**Figure 4.13:** Effects of NAC or 8-Br-cGMP on midgut infection rates of *T. b. brucei* when *G. m. morsitans* were fed on *E. coli* 3 d before infection with trypanosomes. Data presented as the mean  $\pm$  S.E.M from three experiments.  $\chi^2$  tests: \*\*\*  $p < 0.001$  versus the corresponding saline control values.

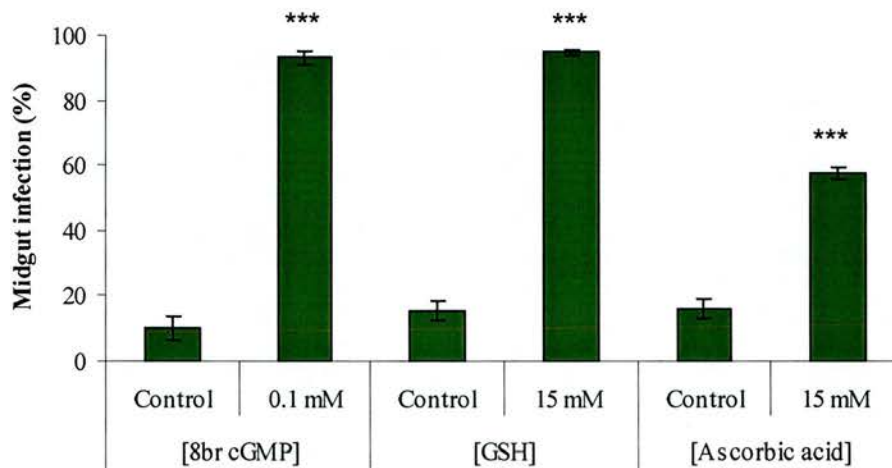
The addition of *E. coli* to the bloodmeal before the infective feed reduced midgut infection rates from a control value of 14% (n=138) to 1% (n=137;  $\chi^2=17.3$ ,  $p < 0.001$ ). If the infective bloodmeal was supplemented with 1 mM NAC the midgut infection rate was reduced in those initially fed *E. coli* from a control value of 57% (n=99) to 24% (n=98;  $\chi^2=21.0$ ,  $p < 0.001$ ). If the infective bloodmeal was supplemented with 5 mM NAC the midgut infection rate was reduced in those initially fed *E. coli* from a control value of 98% (n=105) to 58% (n=99;  $\chi^2=21.0$ ,  $p < 0.001$ ).

The addition of *E. coli* to the bloodmeal before the infective feed reduced midgut infection rates from a control value of 14% (n=141) to 2% (n=133;  $\chi^2=14.9$ ,  $p < 0.001$ ). If the infective bloodmeal was supplemented with 50  $\mu$ M 8-Br-cGMP the midgut infection rate was reduced in those initially fed *E. coli* from a control value of 73% (n=100) to 13% (n=89;  $\chi^2=67.4$ ,  $p < 0.001$ ). If the infective bloodmeal was supplemented with 100  $\mu$ M 8-Br-cGMP the midgut infection rate was reduced in those initially fed *E. coli* from a control value of 84% (n=100) to 36% (n=91;  $\chi^2=45.7$ ,  $p < 0.001$ ).

#### 4.2.14 The effect of 8-Br-cGMP, GSH or ascorbic acid on midgut infection rates of *T. b. rhodesiense* in *G. m. morsitans*

See Section 2.20 for methods.

The effects on midgut infection rates in *G. m. morsitans* of *T. b. rhodesiense* DO when the bloodmeal was supplemented with 8-Br-cGMP, GSH or ascorbic acid are shown in Figure 4.14.



**Figure 4.14:** Effects of 8-Br-cGMP, GSH or ascorbic acid on midgut infection rates of *T. b. rhodesiense* in *G. m. morsitans*. Data presented as the mean  $\pm$  S.E.M from three experiments.  $\chi^2$  tests: \*\*\*  $p < 0.001$  versus the corresponding control values.

The addition of 8-Br-cGMP, GSH and ascorbic acid all significantly increased midgut infection rates of DO in male *G. m. morsitans*. 100  $\mu$ M 8-Br-cGMP increased midgut infection rates from a control value of 10% ( $n=106$ ) to 93% ( $n=103$ ;  $\chi^2=143.4$ ,  $p < 0.001$ ). 15 mM GSH increased midgut infection rates from a control value of 16% ( $n=103$ ) to 95% ( $n=101$ ;  $\chi^2=130.2$ ,  $p < 0.001$ ). 15 mM ascorbic acid increased midgut infection rates from a control value of 16% ( $n=100$ ) to 58% ( $n=95$ ;  $\chi^2=36.9$ ,  $p < 0.001$ ).

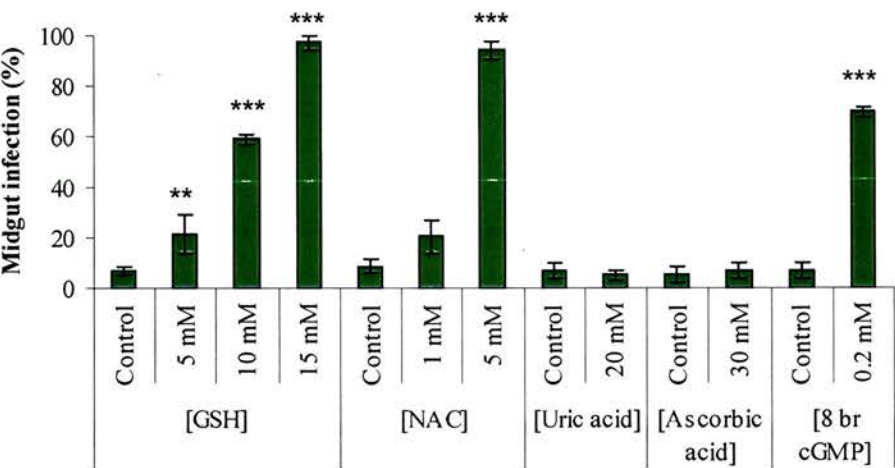
The addition of 8-Br-cGMP and uric acid significantly increased midgut infection rates of DO in female *G. m. morsitans*. The addition of 100  $\mu$ M 8-Br-cGMP increased midgut

infection rates from a control value of 22% (n=60) to 89% (n=47;  $\chi^2=48.3$ ,  $p<0.001$ ). While the addition of 20mM uric acid increased midgut infection rates from a control value of 6% (n=63) to 52% (n=67;  $\chi^2=32.6$ ,  $p<0.001$ ).

#### 4.2.15 The effects of GSH, NAC, uric acid, ascorbic acid or 8-Br-cGMP on midgut infection rates of *T. b. brucei* in *G. p. palpalis*

See Sections 2.22 and 2.23 for methods

The effects on midgut infection rates in *G. p. palpalis* of *T. b. brucei* BUT 135 when the bloodmeal was supplemented with GSH, NAC, uric acid, ascorbic acid and 8-Br-cGMP are shown in Figure 4.15.



**Figure 4.15: Effects of GSH, NAC, uric acid, ascorbic acid or 8-Br-cGMP on midgut infection rates of *T. b. brucei* on *G. p. palpalis*.** Data presented as the mean ± S.E.M from three experiments.  $\chi^2$  tests: \*\*\*  $p<0.001$ ; \*\*  $p<0.01$  versus the corresponding control values.

The addition of 5 mM, 10 mM or 15 mM GSH increased midgut infection rates of BUT 135 in female *G. p. palpalis* from a control value of 7% (n=170) to 21% (n=75), 59%



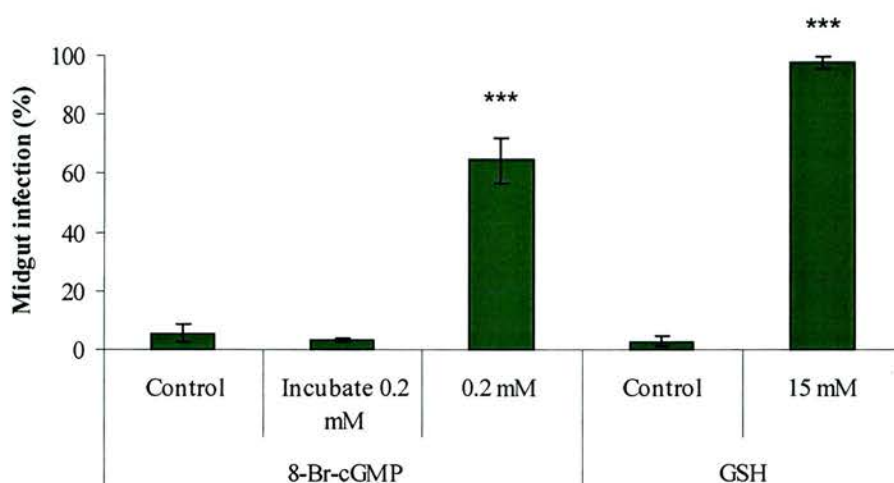
(n=73) and 97% (n=79) respectively (5 mM:  $\chi^2=10.47$ ,  $p=0.001$ ; 10 mM:  $\chi^2=78.4$ ,  $p<0.001$ ; 15 mM:  $\chi^2=192.0$ ,  $p<0.001$ ). The addition of 1 mM or 5 mM NAC increased midgut infection rates of BUT 135 in female *G. p. palpalis* from a control value of 9% (n=70) to 21% (n=68) and 94% (n=71) respectively ( $\chi^2=78.4$ ,  $p<0.001$ ). The addition of 20 mM uric acid resulted in infection rates of 5% (n=77) which was not significantly different from the control value of 7% (n=85;  $\chi^2=0.2$ ,  $p=0.622$ ). The addition of 30 mM ascorbic acid resulted in infection rates of BUT 135 in female *G. p. palpalis* of 7% (n=112) which was not significantly different from the control value of 5% (n=111;  $\chi^2=0.3$ ,  $p=0.592$ ). The addition of 200  $\mu$ M 8-Br-cGMP increased midgut infection rates of BUT 135 in female *G. p. palpalis* from a control value of 7% (n=122) to 70% (n=118;  $\chi^2=100.0$ ,  $p<0.001$ ).

The addition of 200  $\mu$ M 8-Br-cGMP to the infective feed containing DO increased midgut infection rates in male *G. p. palpalis* from a control value of 7% (n=123) to 63% (n=99)

#### **4.2.16 The effects of 8-Br-cGMP or GSH on midgut infection rates of procyclic *T. b. brucei* in *G. m. morsitans***

See Section 2.19 for methods.

The effects on midgut infection rates in *G. m. morsitans* of procyclic *T. b. brucei* BUT 135 with either adding 200  $\mu$ M 8-Br-cGMP to the bloodmeal or by incubating procyclics for 1 h with 200  $\mu$ M 8-Br-cGMP or the addition of 15 mM GSH are shown in Figure 4.16.



**Figure 4.16: Effects of 8-Br-cGMP or GSH on midgut infection rates of procyclic *T. b. brucei* on *G. m. morsitans* .** Data presented as the mean  $\pm$  S.E.M from three experiments.  $\chi^2$  tests: \*\*\*  $p < 0.001$  versus the corresponding control values.

The addition of 200  $\mu$ M 8-Br-cGMP when added directly to the infective bloodmeal containing procyclic *T. b. brucei* BUT 135 increased midgut infection rates from a control value of 6% (n=126) to 65% (n=113;  $\chi^2=93.3$ ,  $p < 0.001$ ). Incubating procyclics with 200  $\mu$ M 8-Br-cGMP for 1 h produced midgut infection rates of 3% (n=124) which was not significantly different from the control ( $\chi^2=0.8$ ,  $p=0.369$ ). Increasing the incubation time to 24 h had no significant effect on infection rates (data not shown). The addition of 15 mM GSH to the infective bloodmeal increased midgut infection rates from a control value of 3% (n=108) to 98% (n=109;  $\chi^2=197.5$ ,  $p < 0.001$ ).

#### 4.2.17 Compounds that had no effect on midgut infection rates

The compounds in Table 4.1 failed to have a significant effect on midgut infection rates of *T. b. brucei* in *G. m. morsitans*.

	MG%	n	± S.E.M	$\chi^2$	<i>p</i>
Control	15	94	3.947502		
10 mM Cystine	10	98	2.375086	=1.0	=0.326
Control	14	92	4.552188		
2 mg Catalase	11	81	2.678571	=0.4	=0.552
Control	6	98	0.625726		
2 mg Superoxide dismutase	6	106	1.508616	<0.1	=0.888
Control	11	82	1.17691		
8 mM Trypanothione	13	87	3.180073	=0.4	=0.544
Control	9	90	2.996861		
20 mM Glutamic acid	11	98	3.680112	=0.3	=0.595
Control	12	91	4.228746		
1 mM Serotonin	13	97	0.262732	<0.1	=0.787
Control	22	67	7.577132		
15 mM Ornithine	17	66	9.166667	=0.7	=0.405
Control	14	97	3.480737		
1 mM EDTA	16	94	0.976986	<0.1	=0.769
10 mM EDTA	20	89	3.262887	=1.1	=0.296
1 mM Nitrilotriacetic acid	16	82	3.934303	<0.1	=0.791
Control	17	58	8.774038		
15 mM Aminoguanidine	13	52	8.030303	=0.3	=0.584

Table 4.1 Compounds which had no effects on midgut infection rates of *T. b. brucei* in *G. m. morsitans*

#### 4.2.18 Summary of compounds having a positive effect on midgut infection rates of *T. b. brucei* in male *G. m. morsitans*

Table 4.2 shows a summary of the compounds which increased infection rates of *T. b. brucei* in male *G. m. morsitans*.

Compound	Dose	MG%	<i>p</i>	Section
<b>GSH</b>	10 mM	97	<0.001	4.2.1
	5 mM	44	<0.001	
	Control	15		
<b>L- or D-Cysteine</b>	10 mM L	100	<0.001	4.2.2
	10 mM D	100	<0.001	
	Control	21		
<b>NAC</b>	1 mM	100	<0.001	4.2.3
	0.5 mM	39	<0.001	
	Control	17		
<b>Ascorbic acid</b>	20 mM	71	<0.001	4.2.4
	10 mM	64	<0.001	
	Control	14		
<b>Uric acid</b>	20 mM	60	<0.001	4.2.5
	10 mM	50	<0.001	
	1 mM	22	=0.036	
	Control	11		
<b>8-Br-cGMP</b>	100 µM	92	<0.001	4.26
	10 µM	51	<0.001	
	Control	16		
<b>cGMP</b>	1 mM	81	<0.001	4.27
	Control	7		

Table 4.2 Summary of compounds having a positive effect on midgut infection rates of *T. b. brucei* in male *G. m. morsitans*.

## 4.3 Discussion

Trypanosomes are transferred between mammalian hosts by the tsetse fly and the first step in this process involves establishing an infection in the midgut of the fly. Several factors have been shown to be important in determining the outcome of a midgut infection, blocking of midgut lectins with specific sugars (Maudlin and Welburn, 1987) and the removal of serum from the bloodmeal (Maudlin *et al.*, 1984) all increase midgut infection rates of in *G. m. morsitans*. The current work shows that the oxidative state of the midgut can also play an important role in killing incoming trypanosomes.

### 4.3.1 The effects of antioxidants on midgut infection rates of *T. b. brucei* in *G. m. morsitans*

The results presented in the current work suggest that trypanosomes can be killed by ROS in the gut of the tsetse fly. The antioxidants GSH, cysteine, NAC, uric acid and ascorbic acid all promote establishment of midgut infections in the tsetse fly *G. m. morsitans*. Studies in *R. prolixus* have shown that, during the breakdown of the bloodmeal hemin, iron and haemoglobin are produced (Souza *et al.*, 1997). These molecules promote oxidative stress by driving Fenton reactions producing hydroxyl ions due to the oxidation of ferrous iron by hydrogen peroxide (Park and Imlay, 2003). The hydroxyl ions produced are cytotoxic and rapidly oxidise cellular components, including lipids and DNA (Gutteridge and Smith, 1988). Hydrogen peroxide has been associated with the trypanocidal properties of Cape buffalo (*Syncerus caffer*) serum due to a reduction in serum catalase levels, resulting in increased levels of hydrogen peroxide in the blood which kills trypanosomes (Wang *et al.*, 1999). Trypanosomes lack the typical antioxidant defences seen in other eukaryotic cells and instead rely on the molecule trypanothione to provide an intracellular reducing environment. Trypanothione is assembled from two molecules of GSH (L-cysteine, glutamic acid and glycine)

conjugated by the polyamine spermidine (Fairlamb *et al.*, 1985). Although L- and D-cysteine possess the same reducing powers they are set apart in that only L-cysteine can be used in the synthesis of proteins such as GSH (Glazenburg *et al.*, 1984; Duszenko *et al.*, 1992). In the present work both L- and D-cysteine were found to promote similar levels of midgut infections so it is unlikely that the effect seen with the two isomers is a result of synthesis of trypanothione, or any other proteins, by the parasite. Although cysteine is the limiting factor in GSH synthesis, to examine other compounds involved in the synthesis of trypanothione, glutamic acid and ornithine (a precursor to spermidine) were added to the infective bloodmeal but no significant effects were observed. GSSG also increased the susceptibility of tsetse to infection with trypanosomes suggesting that there is a turnover of GSH. GSSG is reduced in insects non-enzymatically by thioredoxin reductase (Kanzok *et al.*, 2001) but since in the current work trypanothione failed to increase midgut infections it is likely that trypanothione is not recycled by insect thioredoxin reductase.

Midgut infection rates in *G. m. morsitans* obtained with the same concentrations of GSH or cysteine were similar; however, 10-fold less NAC was required to obtain the same infection rates. NAC has the ability to cross cell membranes while GSH is impermeable to cell membranes (Abello *et al.*, 1994; Laragione *et al.*, 2003). Since 1 mM NAC can promote 100% midgut infection while 10 mM GSH is required to produce the same effects it would seem likely that protecting the intracellular components of the trypanosome is important. This could be due to diffusion of ROS across the trypanosome membrane; GSH would not be able to protect the cell contents from ROS but permeable NAC would. Using RNAi to reduce  $\gamma$ -glutamylcysteine synthetase levels has been shown to be lethal to trypanosomes in culture; however, supplementation with GSH restored intracellular thiol levels (Huynh *et al.*, 2003). This suggests that trypanosomes in the fly midgut can take up GSH and cysteine which could then directly reduce the intracellular environment. Alternatively trypanosomes could move quite quickly to the ectoperitrophic space after entry into the fly gut where they would not have access to the reducing powers of GSH whereas NAC would still be accessible.

The addition of either uric acid or ascorbic acid also resulted in an increase in midgut infection rates; however, 100% infection rates could not be obtained in *G. m. morsitans* with either uric or ascorbic acids. Uric acid is poorly soluble and therefore may not have been fully dissolved in the infective bloodmeal (Kiyohara *et al.*, 1999); however, no such problems were encountered with ascorbic acid. In response to a bloodmeal the concentration of uric acid in the haemolymph of *R. prolixus* rises to ~5 mM and it is thought that this helps prevent damage caused by pro-oxidants produced during digestion. Uric acid is the main solid constituent of tsetse urine (Bursell, 1965; Moloo, 1978) and after bloodmeal ingestion uric acid levels rise substantially over the 3 d it takes to digest a bloodmeal (Brown *et al.*, 1973). Uric acid enters cells via purine nucleobase transporters; however, the H2 transporter found in bloodstream trypanosomes does not take up uric acid (Wallace *et al.*, 2002). Serum concentrations of uric acid are usually in the range of 160-450  $\mu$ M (Ames *et al.*, 1981) and it would be important that the high concentrations of uric acid would not interfere with the transport of other purines as trypanosomes cannot utilise uric acid for purine synthesis (Wallace *et al.*, 2002). The H2 transporter is not found in procyclic trypanosomes and instead the H1 transporter specifically uptakes hypoxanthine, adenine and guanine (de Koning and Jarvis, 1997). Although the H1 transporter was not tested with uric acid (de Koning and Jarvis, loc. cit.), it would seem likely that the H1 transporter found in procyclics would not take up uric acid either, given the high concentrations found in tsetse urine (Bursell, 1965; Moloo, 1978). Trypanosomes and other protozoa do not possess xanthine oxidase and therefore they cannot synthesise uric acid (de Koning and Dhalluin, 2000) and as they cannot transport uric acid, it is doubtful whether uric acid affects trypanosome survival in tsetse via trypanosome metabolism. Rather it appears that the effect of uric acid is to detoxify free radicals within the tsetse gut thereby increasing trypanosome survival.

Ascorbic acid is found at high levels in *Drosophila* even when it is not provided in the diet, suggesting that it plays an important role in insect metabolism (Massie *et al.*, 1991). Larval stages of the Lepidopteran *Orgyia leucostigma* have been shown to possess high levels of ascorbic acid in the gut to detoxify ROS produced in response to feeding



(Barbehenn *et al.*, 2001; Barbehenn *et al.*, 2003). It is likely that the addition of ascorbic acid to the diet of tsetse similarly reduced the effects of ROS produced during bloodmeal breakdown. *T. cruzi* has been shown to possess high levels of ascorbic acid (Clark *et al.*, 1994) and was the first non-photosynthetic organism to be found with an ascorbate-dependent hemoperoxidase (Wilkinson *et al.* 2002a). An enzyme has been found within *T. brucei* which catalyses the formation of ascorbic acid, however, a double gene knockout had no effect on viability of bloodstream forms, suggesting that it is either not essential to survival or that *T. brucei* possesses a transporter which is sufficient to compensate for blockage of the biosynthetic pathway (Wilkinson *et al.*, 2004).

Topoisomerases are enzymes essential for the synthesis and maintenance of nucleic acids and it has been suggested that topoisomerases could act either as targets of ROS generated by Fenton systems or bind the corresponding metal ions, whose redox cycling would generate ROS *in-situ* (Podesta *et al.*, 2004). Topoisomerases from *T. cruzi* and *Crithidia fasciculata* have been shown to be sensitive to hydrogen peroxide (Podesta *et al.*, 2003). Inhibition of topoisomerases would reduce the efficiency of DNA synthesis, a mechanism essential to maintain a parasite population and infection in the tsetse fly. Experiments by Hao *et al.* (2003) reported that NO was not involved in trypanosome death in the tsetse fly; the addition of L-arginine or L-NAME to the infective bloodmeal had no effect on midgut levels of trypanosome infection. This was not the case in the mosquito *A. stephensi* where provision of L-arginine significantly reduced rates of *Plasmodium* parasites and L-NAME increased parasite infections (Luckhart *et al.*, 1997). NO levels were also found to be independent of tsetse fly age (Hao *et al.*, 2003) which, given the greatly increased susceptibility of teneral flies (Welburn and Maudlin, 1992), suggests that NO does not play a role in the refractory nature of tsetse flies. In the current work the NOS inhibitor aminoguanidine did not have a significant effect on midgut infections rates, further supporting the suggestion that NO does not play a role in the outcome of midgut infections.

Iron released during the digestion of bloodmeals can promote the formation of free radicals and to reduce the stress involved in blood feeding *R. prolixus* has been shown to



produce a heme binding protein (Oliveira *et al.*, 1995). This protein, present in the haemolymph, inhibits heme-dependent peroxidation by reducing the amount of free iron available. The mosquito *Aedes aegypti* possesses a ferritin found in the haemolymph which chelates iron (Geiser *et al.*, 2003). In the present study the addition of the iron chelators EDTA and nitrilotriacetic acid had no effect on midgut infection rates of trypanosomes suggesting that free iron does not play a role in susceptibility to infection. Both catalase and superoxide dismutase failed to effect midgut infection rates; however, both these enzymes may simply have been inactivated in the tsetse midgut by the action of trypsin or by low pH.

#### **4.3.2 The effects of cyclic nucleotides on midgut infection rates of *T. b. brucei* in *G. m. morsitans***

Several studies have shown the importance of the second messenger cAMP in the *in vitro* transformation of bloodstream form trypanosomes from long slender to short stumpy forms (Vassella *et al.*, 1997). There is also evidence that adenylate cyclases are activated during transformation of bloodstream forms to procyclics (Rolin *et al.*, 1993). In the current work addition of cAMP to the infective bloodmeal had no significant effect on midgut infection rates. Although the addition of 100  $\mu$ M 8-Br-cAMP nearly doubled infection rates, the difference was not significant when compared to control flies, suggesting that cAMP signalling does not play a role in establishment of midgut infections. By comparison, the addition of cGMP to the blood meal was shown to have a major effect on establishment of trypanosome infections in the midgut of the tsetse fly. As little as 10  $\mu$ M 8-Br-cGMP significantly increased midgut infection rate from 16% to 51%, compared to 10  $\mu$ M 8-Br-cAMP which had no significant effect. So far there have been very few studies on cGMP signalling in kinetoplastids; however, recently a protein kinase A from *T. brucei* has been shown to function through cGMP (Shalaby *et al.*, 2001). An NO transduction pathway has been shown to function in *T. cruzi* (Paveto *et al.*, 1995) and the NO precursor arginine enhances epimastigote motility in *T. cruzi*, an

effect mimicked by the guanylate cyclase activator sodium nitroprusside and by 8-Br-cGMP (Pereira *et al.*, 1997). In the present work the addition of sodium nitroprusside to the infective bloodmeal was toxic to tsetse flies within 4 d post-infection at low micromolar concentrations (data not shown) probably through the release of cyanide (Ruan *et al.*, 1999).

The current work shows that cGMP could play an important role in signalling in trypanosomes. The enzyme soluble guanylate cyclase can be activated by the binding of NO (Bellamy *et al.*, 2002) and in mammalian cells NO can act as an anti-apoptotic factor mediated downstream by protein kinase G; 8-Br-cGMP is a direct protein kinase G activator preventing activation of pro-apoptotic pathways (Fiscus, 2002). The anti-apoptotic effects of cGMP have been demonstrated in the present work by its ability to rescue dying midgut trypanosome infections up to 4 d after trypanosomes have entered the fly midgut, whereas GSH was only able to rescue infections within 2 d. This suggests that trypanosomes are normally killed or receive a signal to die within 2 d of entering the midgut. Since the death process can be reversed with the addition of 8-Br-cGMP it seems likely that trypanosomes enter an apoptotic state in the tsetse gut which can be overridden up to 4 d post-infection. To examine whether trypanosomes had moved into the ectoperitrophic space and would not have access to GSH, 5mM NAC was added to the bloodmeal 72 h post-infection. Since NAC has the ability to cross cell membranes (Abello *et al.*, 1994; Laragione *et al.*, 2003) it would be able to pass easily into the ectoperitrophic space where the trypanosomes reside after establishing an infection. Since there was no significant difference between control flies and those fed 5 mM NAC 72 h post-infection it would seem likely that even if trypanosomes had entered the ectoperitrophic space they could not be rescued from the death process by the addition of a cell permeable antioxidant of this type.

#### **4.3.3 The effects of antioxidants or 8-Br-cGMP on non-teneral *G. m. morsitans* infected with *T. b. brucei***

Tsetse flies which have received a bloodmeal before the infective bloodmeal show greater refractoriness to infection than teneral flies infected at their first feed (Welburn and Maudlin, 1992). In the present work, non-teneral flies infected with either 100  $\mu$ M 8-Br-cGMP or 10 mM GSH did not produce midgut infections comparable to teneral flies treated in the same way. However, increasing the concentration of 8-Br-cGMP or GSH to 200  $\mu$ M and 15 mM respectively boosted midgut infection to >90%. This was also the case for flies fed NAC with 5 mM NAC required to obtain >95% in fed flies while teneral flies could be 100% infected with 1 mM NAC. The addition of 20 mM ascorbic acid also significantly increased midgut infection rates in non-teneral flies but concentrations of ascorbic acid above 20 mM had a toxic effect on tsetse and therefore could not be tested (data not shown). The addition of 20 mM uric acid had no effect on infection rates in non-teneral flies; increasing the dose of uric acid had an anti-feedant effect on the flies so higher concentrations could not be tested.

The addition of 8-Br-cGMP or GSH at the first feed followed by infection at the second feed significantly increased trypanosome infection rates which is similar to results obtained with glucosamine (Welburn and Maudlin, 1992), suggesting that both compounds have a residual effect on infection rates.

#### **4.3.4 The effects of *E. coli* and *S. glossinidius* on midgut infection rates of *T. b. brucei* in *G. m. morsitans***

Previous experiments by Hao *et al.* (2001) have shown that injection of *E. coli* into the haemolymph of tsetse flies reduces susceptibility to trypanosome infection. Although the infective feed was supplemented with D+ glucosamine in this work, injection of *E. coli* significantly reduced the number of flies with midgut infections (Hao *et al.*, loc. cit.).

In the current work injection of *E. coli* into the haemolymph resulted in a significant decrease in the number of midgut infections when the infective bloodmeal was supplemented with 1 mM NAC. However, increasing the concentration of NAC to 5 mM overcame the barriers to infection when the fly was injected with *E. coli*.

When 50  $\mu$ M 8-Br-cGMP was added to the infective feed there was no significant difference between the midgut infection rates between control flies and those injected with *E. coli*. This suggests that the immune response of the tsetse stimulated by injection of *E. coli* is not able to override the effect of 8-Br-cGMP on trypanosome midgut infection rates or that 8-Br-cGMP overrides the tsetse immune response.

When flies were injected with *S. glossinidius* no significant difference was found between control flies and those exposed to either *S. glossinidius* from *G. m. morsitans* or *S. glossinidius* from *G. p. palpalis*. This would suggest that increasing the levels of *S. glossinidius* in the fly does not affect the immune status of the fly. *S. glossinidius* from *G. m. morsitans* has been injected into *G. p. palpalis* and found to be inherited by the offspring (Cheng and Aksoy, 1999). 16S rDNA from *S. glossinidius* derived from different tsetse species are identical (Chen *et al.*, 1999) suggesting that injection of *S. glossinidius* from different tsetse would not affect the immune status of the fly.

In the current work feeding of *E. coli* in the bloodmeal before the infective bloodmeal significantly reduced midgut infections whether the bloodmeal was supplemented with NAC or 8-Br-cGMP, while flies fed *E. coli* and infected with an unsupplemented bloodmeal produced midgut infections of <2%. Although midgut infections were lower in those that had ingested *E. coli* rather than injected *E. coli* this is probably due to the teneral phenomenon with flies which have not fed being easier to infect than fed flies (Welburn and Maudlin, 1992). However, the lower infection rates obtained when *E. coli* was ingested could be due to interaction of the bacteria with the gut environment invoking a greater trypanocidal effect when the next bloodmeal is taken.

These results show that pre-immune stimulation of the tsetse fly significantly reduces the number of midgut infections when *E. coli* is both injected (when the infective feed contains NAC) and fed to flies (when the infective feed contains either NAC or 8-Br-cGMP). Ingestion of *E. coli* before the infective bloodmeal significantly reduced the

prevalence of *Brugia malayi* (Lowenberger *et al.*, 1996) and *Plasmodium* (Lowenberger *et al.*, 1999) infections in mosquitoes.

#### **4.3.5 The effect of the sex of the fly on midgut infection rates of *T. b. brucei* in *G. m. morsitans***

No significant differences were found in midgut infection rates between male and female flies suggesting that the two sexes have the same levels of susceptibility. This was independent of the nature of the infective feed, supplementation of the bloodmeal with GSH, uric acid or 8-Br-cGMP produced similar midgut infection rates in both male and female flies. This agrees with the results of Maudlin *et al.* (1986) and Welburn and Maudlin (1992) who found no significant differences in midgut infection rates between male and female flies that were susceptible to trypanosome infection.

#### **4.3.6 The effects of antioxidants and 8-Br-cGMP on midgut infection rates of *T. b. rhodesiense* in *G. m. morsitans***

The addition of GSH, ascorbic acid, uric acid and 8-Br-cGMP to the infective feed also increased midgut infection rates of *T. b. rhodesiense* (strain DO) showing that the addition of antioxidants and cGMP to the bloodmeal and the subsequent increase in infection rates is not restricted to *T. b. brucei*.

#### **4.3.7 The effects of antioxidants and 8-Br-cGMP on midgut infection rates of *T. b. brucei* in *G. p. palpalis***

Riverine tsetse species belonging to the *palpalis* group are innately more refractory to trypanosome infection when compared with savannah flies of the *morsitans* group

(Moloo and Kutuza, 1988a, 1988b; Moloo *et al.*, 1992; Welburn *et al.*, 1994). In the present work greater concentrations of GSH or NAC were required to achieve >95% infections in *G. p. palpalis* when compared to *G. m. morsitans*. Addition of 200  $\mu$ M 8-Br-cGMP increased trypanosome infections rates to 70% whereas in *G. m. morsitans* 100  $\mu$ M 8-Br-cGMP increases infection rates to >95%. The addition of uric and ascorbic acids failed to have any effect on midgut infections in *G. p. palpalis*.

Previous experiments by Welburn *et al.* (1994) showed that the addition of glucosamine did not increase trypanosome infection rates in *G. p. palpalis* past 60% whereas 100% infection rates could be achieved in *G. m. morsitans* at this dose. Increasing the dose of glucosamine had no additional effect on infection rates; instead, the addition of galactose as well as glucosamine was required to increase infection rates to 100% (Welburn *et al.*, loc. cit.). In the present work increasing the dose of GSH or NAC resulted in infection rates of >95%, which suggests that a different mechanism is operating from that involving sugars. The results indicate that there is a higher production of ROS in *G. p. palpalis* compared to *G. m. morsitans* and that ROS produced cannot be detoxified by the addition of uric acid and ascorbic acid. Increased production of ROS could account for the refractory nature of riverine species and suggests that the *palpalis* gut is a more hostile environment to trypanosomes than the *morsitans* gut.

#### **4.3.8 The effects of antioxidants and 8-Br-cGMP on midgut infection rates of procyclic *T. b. brucei* in *G. m. morsitans***

To examine if pre-treatment of trypanosomes with 8-Br-cGMP could influence infection rates, procyclic trypanosomes were incubated with 200  $\mu$ M 8-Br-cGMP for 1 h. No significant differences were found between those incubated with 8-Br-cGMP and control flies. However, when 8-Br-cGMP was added to the infective bloodmeal midgut infection rates rose significantly. This could mean that the cGMP affects the fly immune response in some way or that cGMP stops the process of apoptosis thought to kill trypanosomes set in motion by ROS.

The effect of the addition of 8-Br-cGMP to the bloodmeal 96 h post-infection with bloodstream forms suggests that it is acting on the trypanosome rather than on the fly. Since most trypanosomes die in the first 60 h (Welburn *et al.*, 1989) of infection it is unlikely that turning off some aspect of the fly immune response after 96 h would have any effect and instead it is more likely that 8-Br-cGMP is acting directly on the trypanosomes.

Unlike 8-Br-cGMP, the addition of 10 mM GSH to the infective bloodmeal resulted in an increase in infection rates to close to 100%. This could be due to trypanocidal lectins binding to the procyclic surface coat resulting in trypanosome death before cGMP could override the apoptotic signal. However, addition of 8-Br-cGMP to the bloodmeal 4 d post-infection significantly increased infection rates and bloodstream form trypanosomes rapidly transform to procyclics in the tsetse midgut Turner *et al.* (1988b) found that most trypanosomes had lost their VSG coat and fully replaced it with procyclin within 2-3 h after ingestion by the tsetse fly). Therefore it would seem unlikely that expressing procyclin when entering the midgut would account for the differences in infection rates seen between bloodstream or procyclic forms. The infection rates of the control infections were also lower in the experiments initiated with procyclics which suggests that a factor in mouse blood could play a role in promoting establishment of bloodstream form trypanosomes.

#### **4.3.9 Factors which may affect susceptibility to infection**

The tsetse fly diet consists exclusively of vertebrate blood which needs to be broken down in order to obtain nutrients, a process taking between 2 and 3 d (Langley *et al.*, 1978). From previous work it was thought that initiation of bloodmeal digestion resulted in the secretion of trypanocidal lectins; lectins would bind to the trypanosome surface signalling the trypanosome to die in a process similar to apoptosis (Welburn *et al.*, 1996). The most significant factor in susceptibility to infection of tsetse flies to trypanosomes has been shown to be maternally inherited (Maudlin and Dukes, 1986;



Maudlin *et al.*, 1986). The current work has shown that ROS play an important role in the development of a trypanosome infection and the question arises how this would work in a susceptible stock of tsetse. Susceptible flies might lack the necessary ROS response to kill incoming trypanosomes. It is probable that *S. glossinidius* could induce changes in tsetse redox responses as injection of *E. coli* lipopolysaccharide interferes with GSH and ascorbic acid levels in mice (Victor *et al.*, 2002). In tsetse, changes in uric acid production could affect the outcome of an encounter with trypanosomes; as we have seen, as little as 1 mM uric acid significantly increased midgut infection rates in *G. m. morsitans*. Experiments have shown that levels of uric acid in tsetse flies can be as high as 1.37  $\mu$ M (Brown *et al.*, 1973) although this is three orders of magnitude lower than the amount needed in the bloodmeal to influence infection rates, the measurements were from whole flies rather than gut tissues. It is possible that high levels of uric acid could be found in the tsetse gut close to the junction of the hind gut where the trypanosomes enter the ectoperitrophic space. The other possible source of antioxidants is from the bloodmeal; GSH is found in human serum at concentrations less than 10  $\mu$ M (Smith *et al.*, 1996), ascorbic acid concentrations vary from 20-90  $\mu$ M (Halliwell, 2001) and uric acid is found at concentrations between 160-450  $\mu$ M (Ames *et al.*, 1981). These concentrations are all too low to influence infection rates and, given that the removal of serum from the infective feed increases susceptibility to infection (Maudlin *et al.*, 1984), suggest that serum levels of antioxidants do not play a role in the establishment of midgut infections. Although serum levels of GSH are quite low, intracellular concentrations range from 1-10 mM depending on cell type (Smith *et al.*, 1996), with red blood cells containing concentrations of around 2 mM (Ames *et al.*, 1981). Since red blood cells are broken down during digestion this could release intracellular GSH. However, since all flies receive the same bloodmeal and flies that are infected with serum without red cells produce similar midgut infections after 6 d (Olubayo *et al.*, 1994) it is unlikely that GSH from the host plays a substantial role in the establishment of trypanosome infections. Since soluble guanylate cyclase has been shown to function through NO it is possible that flies which are susceptible to infection do not produce cytotoxic levels of NO.

Instead a restricted NO response low enough to activate guanylate cyclase could lead to trypanosome establishment in the tsetse gut. Hao *et al.* (2003) investigated NO levels in response to infection with trypanosomes 24 h after infection and although they found that infection with trypanosomes led to a decrease in NO concentrations this result was taken from pooled samples of 25 flies. However, as these NO readings were taken at 24 h post-infection (Hao *et al.*, 2003) it would be impossible to predict if the trypanosomes would have gone on to develop a midgut infection. Given the midgut infection rates of ~50% obtained by Hao *et al.* (2003), it could be that differences in flies that were susceptible and refractory would not be picked up in pooled samples. Experiments investigating NO levels in response to trypanosome infections using susceptible and refractory lines of tsetse would be informative.

#### **4.3.10 Conclusions**

In conclusion the addition of antioxidants and cGMP all significantly increased the susceptibility of *G. m. morsitans* to infection with trypanosomes. *G. p. palpalis* which is intrinsically more refractory to infection required higher concentrations of cGMP, GSH and NAC while ascorbic acid and uric acid had no effect in this species. This would suggest that higher levels of ROS are produced in riverine flies and could explain why they show lower levels of infection than savannah flies in both the laboratory and field. The current work suggests that antioxidants work by detoxification of ROS in the fly midgut rather than the production of proteins, shown through the use of the cysteine isomers; however, the action of cGMP is still not fully understood. Although pre-treatment of procyclics with cGMP did not result in increases in infection this does not necessarily mean that the cGMP is working via the fly. cGMP may override a signal received by the trypanosome in the fly which would normally lead to apoptosis in the trypanosome; providing an anti-apoptotic signal to trypanosomes before they receive the signal to die may not affect midgut infections in tsetse flies.

## **Chapter 5**

### **Factors affecting maturation of midgut trypanosome infections in tsetse flies**

## 5.1 Introduction

To complete its lifecycle in the fly, the procyclic trypanosome must exit the peritrophic space in the tsetse midgut and make its way to the salivary gland (*Trypanozoon*) or proboscis (*Nannomonas*) where it matures to form mammalian infective metacyclics. However, populations of tsetse with mature salivary gland infections generally make up less than 1% of the total wild population, for example Okoth and Kapaata (1986) reported that less than 1% of *G. f. fuscipes* from Busoga in Uganda carried a salivary gland infection. While Madubunyi (1987) examined 4620 *G. tachinoides* from Nsukka in Nigeria and found mature infections in 0.65% of tsetse examined; of these mature infections 20% were *T. brucei*, 40% were *T. congolense* and 40% comprised mixed infections of *T. brucei* and *T. congolense*.

### 5.1.1 Factors which influence maturation of midgut infections

Maturation is not an automatic process and is dependent on both fly and trypanosome factors, including temperature, fly species, fly sex, trypanosome genotype, interactions with midgut lectins and serum factors (Molyneux, 1977; Maudlin, 1991; Molyneux and Stiles, 1991).

The number of trypanosomes in the gut is not a determining factor in the outcome of maturation, as similar numbers are found in the midgut of flies with or without mature infections (Maudlin and Welburn, 1997). Van Den Abbeele *et al.* (1999) reported that trypanosomes appeared in the salivary glands after eight days; however, it was not until four days later that they became infective to mice. After day 12 the proportion of flies with salivary gland infections did not increase and all salivary glands that were positive for trypanosome infection were mammalian infective after 18 d (Van Den Abbeele *et al.*, loc. cit). Dale *et al.* (1995) reported that the time taken to mature an infection is not parasite species-specific, with the mean maturation time for trypanosome stocks 1/148

FLY9, SIKUDA88 (both *T. congolense*) and EATRO 2340 (*T. b. rhodesiense*) were found to be 12.5, 15.5 and 18 d respectively. The only significant difference in maturation rates was found to be between 1/148 FLY9 and EATRO 2340.

Higher rates of maturation are achieved in tsetse flies with trypanosome stocks that mature earlier; the longer a trypanosome strain takes to mature its first mature infection the lower the final transmission rate will be (Milligan *et al.*, 1995). Welburn and Maudlin (1997) suggested that there was a short window of opportunity in which trypanosomes had to mature; if the maturation process had not begun by 11 d post-infection the trypanosome had effectively missed its chance to mature.

Both the tsetse and the trypanosome use proline as their main energy source and although it has been estimated that infected tsetse would lose 15% of their flight time, it is difficult to test the effect of energy loss in the wild on the tsetse in the lab as they are kept under defined conditions and are provided with blood meals at least every couple of days (Bursell, 1981). In the wild the tsetse would be under pressure to find a bloodmeal and a loss of 15% of its flight time could have important implications on transmission of trypanosomes. However, parasite invasion of the salivary glands has a detrimental effect on the survival of the tsetse fly. Experiments by Maudlin *et al.* (1998) showed that salivary infections significantly increased tsetse mortality although this effect only becomes noticeable 50 d post-infection. With the average lifespan in the wild of male and female tsetse being 24 d and 35 d respectively (Welburn and Maudlin, 1999), it is unlikely that death induced by salivary gland infections plays a role in wild tsetse. Although it has been possible to create lines of flies susceptible to midgut infection, it has not been possible to select for maturation, however, this may simply be due to technical difficulties (Ian Maudlin, personal communication).

#### **5.1.1.1 Temperature**

Increased temperatures have been shown to potentiate transmission of trypanosomes through tsetse. *T. vivax* will complete development in 5 d in *G. p. palpalis* incubated at

29°C compared to 12-13 d at 22°C (Desowitz and Fairbairn, 1955) while increasing the incubation temperature to 31°C results in higher rates of transmission of *T. brucei* in *G. m. morsitans* (Dipeolu and Adam, 1974). Although *T. b. rhodesiense* could establish midgut infections in *G. morsitans* at 15°C, no salivary gland infections developed until the incubation temperature was increased to 26°C (Kinghorn and Yorke, 1913). Maturation of *T. b. brucei* in *G. m. morsitans* is also completely blocked at 20°C (Dipeolu and Adam, 1974). Otieno *et al.* (1983) suggested that a cold shock (0-5°C for 30 min) administered immediately after the infective bloodmeal increased maturation rates of *T. b. brucei* in male *G. m. morsitans*.

#### 5.1.1.2 Fly species

Although it is possible to infect tsetse flies with different strains of trypanosomes it is thought that some tsetse/trypanosome relationships result in higher rates of transmission. Generally, flies of the *morsitans* group will mature a higher proportion of midgut infections than flies of the *palpalis* group. Experiments have shown that *G. m. morsitans* will mature all established midgut infections with some strains of *T. congolense*, whereas *G. p. palpalis* infected with the same trypanosomes will very rarely produce mature infections (Maudlin and Welburn, 1989; Welburn *et al* 1994). Moloo and Kutuza (1988a) investigated the vectorial capacity of seven tsetse species and found that *G. m. centralis* was the most efficient vector of isolates of *T. b. brucei* from both Tanzania and Nigeria. Although *G. brevipalpis* was highly susceptible to midgut infection with the *T. b. brucei* from Tanzania (midgut infection rate=59%) only one fly matured an infection (transmission of infectivity=0.4%), suggesting that it is a very poor vector of trypanosomes. Midgut infection rates in the *palpalis* group of flies were too low for any comparisons to be made although *G. tachinoides* did mature a high proportion of established midgut infections (Moloo and Kutuza, loc. cit.).

#### 5.1.1.3 Fly sex

Differences in maturation rates of trypanosomes are usually observed between male and female tsetse involving *Trypanozoon* infections. Burt (1946), Maudlin *et al.* (1990), Dale *et al.* (1995) and Milligan *et al.* (1995) reported that male flies matured proportionally more *Trypanozoon* infections than female flies. However, the time taken for infections to mature in females is the same as males suggesting that female flies do not mature infections at a slower rate (Dale *et al.*, 1995). Several reasons have been put forward to explain this phenomenon, which has been suggested to be X linked. Maudlin *et al.* (1990) and Milligan *et al.* (1995) suggested that maturation was controlled by a single sex-linked gene in tsetse, which either killed trypanosomes or stopped them maturing. However, the differences in maturation rates of trypanosomes is restricted to the *Trypanozoon*. Dale *et al.* (1995) found no significant differences between maturation rates in male or female *G. m. morsitans* infected with *T. congolense*, while Moloo *et al.* (1992) found no difference between maturation rates in male or female *G. m. centralis* infected with *T. vivax*.

#### 5.1.1.4 Trypanosome genotype

The capacity to infect humans is offset by a reduction in the ability of the trypanosome to mature in the tsetse fly. Experiments with 37 trypanosome stocks (22 resistant and 15 sensitive to human serum) showed that resistance to human serum resulted in a significant reduction in maturation of the parasite (Welburn *et al.*, 1995). Maturation in male and female flies was reduced by factors of 1.8 and 2.7 respectively, with 27% of the *T. b. rhodesiense* stocks failing to produce a single mature infection in female flies. Significant differences in maturation rates were found between *Trypanozoon* stocks and *T. congolense* (Maudlin and Welburn, 1988). Three different stocks of *T. congolense* matured greater than 83% of established midgut infections compared to rates less than 50% in both *T. b. brucei* and *T. b. rhodesiense* (Maudlin and Welburn loc. cit.)



Moloo *et al.* (1992) showed that *G. m. centralis* had a higher vectorial capacity than *G. pallidipes* for both *T. congolense* and *T. b. brucei*; however, they found no significant difference between *G. m. centralis* and *G. pallidipes* in vectoring *T. vivax*.

#### 5.1.1.5 Lectins

Maturation of midgut infections of trypanosomes has been linked to tsetse midgut lectins; the addition of glucosamine to the tsetse diet is thought to block the action of midgut lectins, resulting in an increased susceptibility to trypanosome infection, however, continuous feeding of D+ glucosamine reduces the maturation rates of established midgut infections of both *T. congolense* and *T. brucei* (Maudlin and Welburn, 1988; Welburn and Maudlin 1989). Experiments have shown that feeding D+ glucosamine for as little as 5 d post-infection significantly reduces maturation in *T. congolense*, suggesting that the signal to mature is produced in the first few days of infection (Welburn and Maudlin, 1989). The addition of the sugar melibiose also reduced proportions of midgut infections which matured; however, unlike glucosamine, melibiose had no effect on potentiating the susceptibility of the fly to infection with trypanosomes (Welburn and Maudlin, 1990). Lectins also play an important role in the transformation of *T. cruzi* within the gut of the vector *R. prolixus* (Pereira *et al.*, 1981).

#### 5.1.1.6 Serum factors

The removal of serum from the diet has been shown to reduce maturation of *T. congolense* in *G. m. morsitans* (Maudlin *et al.*, 1984). The replacement of serum with saline increased the susceptibility to infection with midgut infections increasing from 45% to 91%. However, the proportions of midgut infections that matured were significantly reduced from 45% to 3%. Flies that were fed on a diet lacking lipids matured significantly less infections, proportions of midgut infections maturing were

reduced from 42% to 5%. The addition of  $\gamma$  globulin to the diet consisting of saline and bovine red cells increased the rates of midgut infections maturing from 6% to 21%. From the above experiments, Maudlin *et al.* (loc. cit.) suggested that serum factors particularly lipids were important in the trypanosome maturation process; however, as lipids are insoluble in water, it was impossible to examine the roles they played in the maturation process. It was suggested that serum factors in the bloodmeal resulted in the secretion of lectins which provided a signal to mature (Welburn and Maudlin, 1999).

### **5.1.2 The present work**

In recent years there has been little advancement in our understanding of the maturation process of trypanosomes in tsetse and the present work attempts to address this issue. With the information gleaned from the work presented in the previous chapter the effects of the different antioxidants and the cyclic nucleotides cAMP and cGMP on transmission rates of trypanosomes in *G. m. morsitans* are investigated.

## 5.2 Results

See Section 2.16 for details on trypanosome production, Section 2.17 for details on infection and dissection of tsetse flies and Section 2.26.7 for details on statistical analysis.

### 5.2.1 The effects of one feed of GSH on transmission rates of *T. b. brucei* in *G. m. morsitans*

See Section 2.24.1 for methods.

The effects of the addition of 15 mM GSH to the infective bloodmeal on midgut infection rates and transmission rates of *T. b. brucei* BUT 135 in *G. m. morsitans* are shown in Figure 5.1.

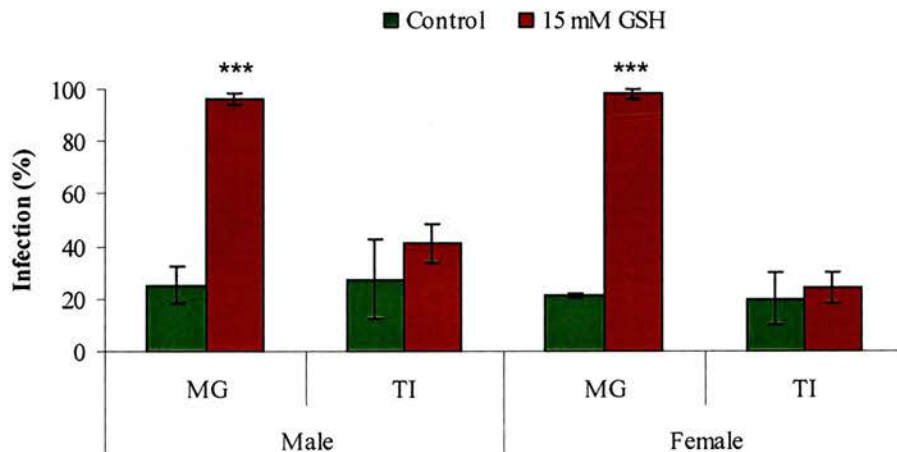


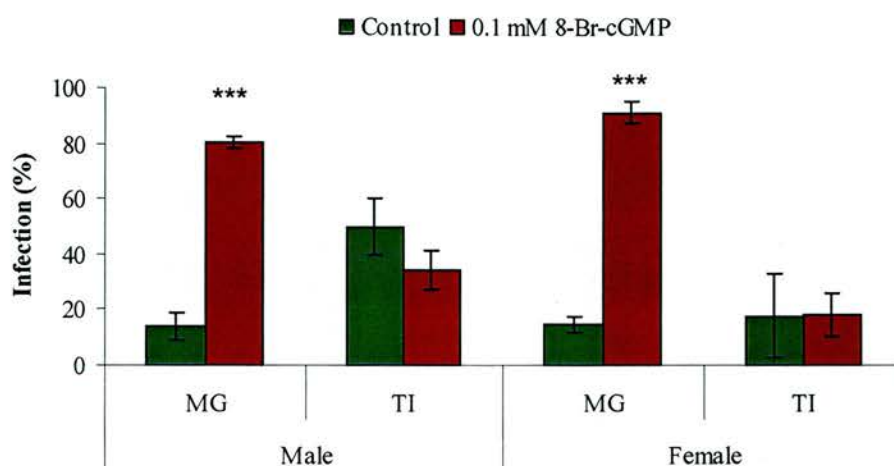
Figure 5.1: Effects of one feed of 15 mM GSH on midgut infection rates and transmission rates of *T. b. brucei* in *G. m. morsitans*. Data presented as the mean  $\pm$  Standard error of mean (S.E.M) from three experiments. Legend: MG=midgut; TI= transmission of infectivity.  $\chi^2$  tests: \*\*\*  $p < 0.001$  versus the corresponding control value.

Tsetse dissected after 28 d showed significant differences in midgut infection rates in both male and female *G. m. morsitans*. Supplementation of the infective feed with 15 mM GSH increased midgut infection from a control value of 25% (n=87) to 96% (n=83) in male flies and from 21% (n=118) to 98% (n=109) in female flies (male:  $\chi^2=89.5$ ,  $p<0.001$ ; female:  $\chi^2=138.0$ ,  $p<0.001$ ). However, there were no significant differences between the transmission rates of control flies and those supplied with 15 mM GSH. Transmission of infectivity (TI) values in male flies were 27% (n=22) in control flies compared to 41% (n=80) in those fed 15 mM GSH ( $\chi^2=1.4$ ,  $p=0.232$ ). TI values in female flies were 20% (n=25) in control flies compared to 24% (n=107) in those fed 15 mM GSH ( $\chi^2=0.2$ ,  $p=0.648$ ).

### **5.2.2 The effects of one feed of 8-Br-cGMP on transmission rates of *T. b. brucei* in *G. m. morsitans***

See Section 2.24.1 for methods.

The effects of the addition of 100  $\mu$ M 8-Br-cGMP to the infective bloodmeal on midgut infection rates and transmission rates of *T. b. brucei* BUT 135 in *G. m. morsitans* are shown in Figure 5.2.



**Figure 5.2: Effects of one feed of 100  $\mu$ M 8-Br-cGMP on midgut infection rates and transmission rates of *T. b. brucei* in *G. m. morsitans*. Data presented as the mean  $\pm$  S.E.M from three experiments. Legend: MG=midgut infection rate; TI= transmission of infectivity.  $\chi^2$  tests: \*\*\*  $p < 0.001$**

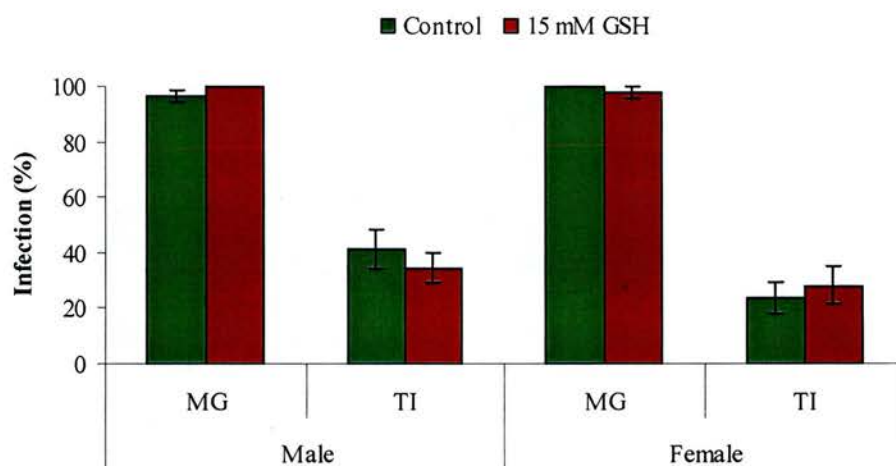
Tsetse dissected after 28 d showed significant differences in midgut infection rates in both male and female *G. m. morsitans*. Supplementation of the infective feed with 100  $\mu$ M 8-Br-cGMP increased midgut infection from a control value of 14% (n=99) to 80% (n=82) in male flies and from 15% (n=115) to 91% (n=90) in female flies (male:  $\chi^2=80.0$ ,  $p < 0.001$ ; female:  $\chi^2=117.8$ ,  $p < 0.001$ ). However, there were no significant differences between transmission rates of control flies and those infected with 100  $\mu$ M 8-Br-cGMP. TI values in male flies were 50% (n=14) in control flies compared to 34% (n=67) in those fed 100  $\mu$ M 8-Br-cGMP ( $\chi^2=1.2$ ,  $p=0.269$ ). TI values in female flies were 18% in both control flies (n=17) and those infected with 100  $\mu$ M 8-Br-cGMP (n=82;  $\chi^2 < 0.1$ ,  $p=0.950$ ).

### 5.2.3 The effects of continually feeding GSH on transmission rates of *T. b. brucei* in *G. m. morsitans*

See Section 2.24.3 for method.

#### 5.2.3.1 Infective bloodmeal containing GSH

The effects of continuously feeding of 15 mM GSH on midgut infection rates and transmission rates of *T. b. brucei* BUT 135 in *G. m. morsitans* are shown in Figure 5.3.



**Figure 5.3:** Effects of continually feeding 15 mM GSH on midgut infection rates and transmission rates of *T. b. brucei* in *G. m. morsitans*. Data presented as the mean  $\pm$  S.E.M from three experiments. Legend: MG=midgut infection rate; TI= transmission of infectivity.

Flies dissected after 28 d showed no significant differences in midgut infection rates in either male or female *G. m. morsitans* with the continual addition of 15 mM GSH. Midgut infection rates in male flies were 96% (n=83) in control flies compared to 100% (n=78) in those continually fed 15 mM GSH ( $\chi^2=2.8$ ,  $p=0.090$ ). Midgut infection rates

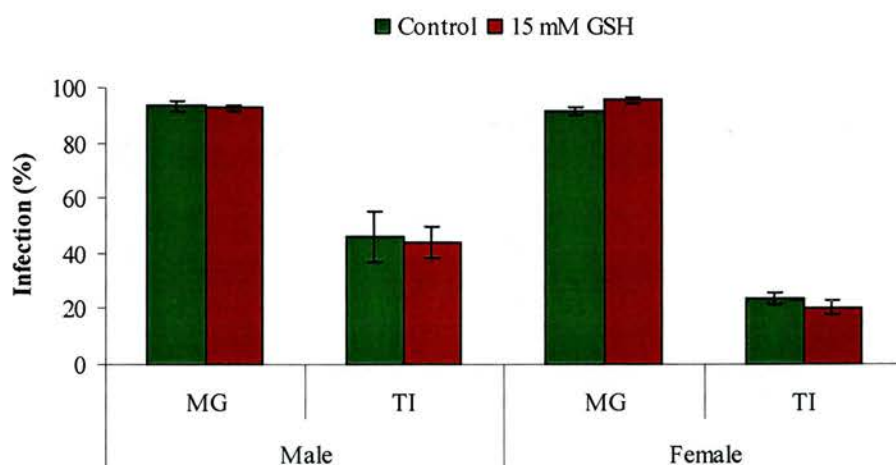


in female flies were 100% (n=101) in control flies compared to 98% (n=94) in those continually fed 15 mM GSH ( $\chi^2=2.2$ ,  $p=0.141$ ). The continual addition of 15 mM GSH to the bloodmeal had no significant effect on transmission rates in either male or female flies. TI values in male flies were 41% (n=80) in control flies compared to 35% (n=78) in those continually fed 15 mM GSH ( $\chi^2=0.7$ ,  $p=0.390$ ). TI values in female flies were 24% (n=101) in control flies compared to 28% (n=92) in those continually fed 15 mM GSH ( $\chi^2=0.5$ ,  $p=0.476$ ).

### 5.2.3.2 Infective bloodmeal containing 8-Br-cGMP

See Section 2.24.2 for method.

The effects of the subsequent addition of 15 mM GSH to the bloodmeal on midgut infection rates and transmission rates of *T. b. brucei* BUT 135 in *G. m. morsitans* when the infective feed was supplemented with 100  $\mu$ M 8-Br-cGMP are shown in Figure 5.4.



**Figure 5.4:** Effects of the subsequent addition of 15 mM GSH to the bloodmeal on midgut infection and transmission rates of *T. b. brucei* in *G. m. morsitans* when the infective feed contained 100  $\mu$ M 8-Br-cGMP. Data presented as the mean  $\pm$  S.E.M from three experiments. Legend: MG= midgut infection rate; TI= transmission of infectivity.



To enhance midgut infections to a level at which changes in rate of maturation would be apparent all tsetse flies received an infective bloodmeal supplemented with 100  $\mu$ M 8-Br-cGMP.

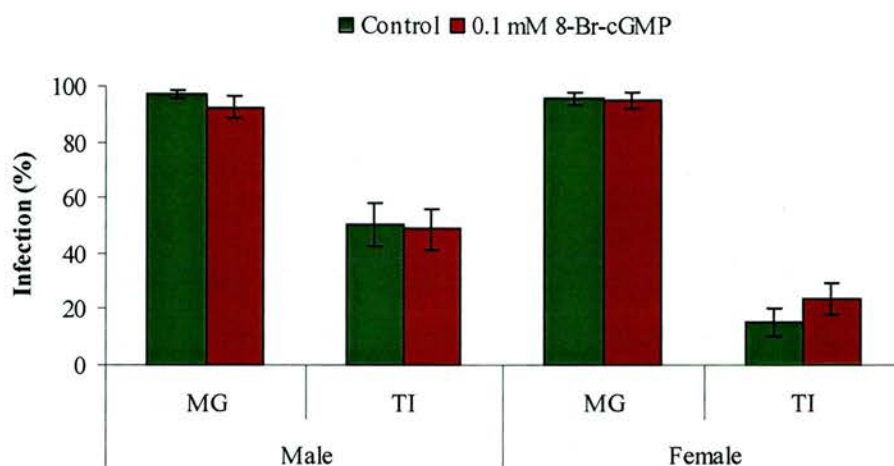
Flies dissected after 28 d showed no significant differences in midgut infection rates in either male or female *G. m. morsitans* with the subsequent addition of 15 mM GSH. Midgut infection rates in male flies were 93% in both control flies (n=91) and those subsequently fed 15 mM GSH (n=98;  $\chi^2 < 0.1$ ,  $p = 0.882$ ). Midgut infection rates in female flies were 92% (n=96) in control flies compared to 95% (n=92) in those continually fed 15 mM GSH ( $\chi^2 = 1.2$ ,  $p = 0.264$ ). The subsequent addition of 15 mM GSH to the bloodmeal had no significant effect on transmission rates in either male or female flies. TI values in male flies were 46% (n=85) in control flies compared to 44% (n=91) in those subsequently fed 15 mM GSH ( $\chi^2 < 0.1$ ,  $p = 0.797$ ). TI values in female flies were 24% (n=88) in control flies compared to 20% (n=88) in those subsequently fed 15 mM GSH ( $\chi^2 < 0.1$ ,  $p = 0.738$ ).

#### **5.2.4 The effects of continually feeding 8-Br-cGMP on transmission rates of *T. b. brucei* in *G. m. morsitans***

##### **5.2.4.1 Infective bloodmeal containing 8-Br-cGMP**

See Section 2.24.2 for method.

The effects of continuously feeding 100  $\mu$ M 8-Br-cGMP on midgut infection rates and transmission rates of *T. b. brucei* BUT 135 in *G. m. morsitans* are shown in Figure 5.5.



**Figure 5.5: Effects of continually feeding 100  $\mu$ M 8-Br-cGMP on midgut infection rates and transmission rates of *T. b. brucei* in *G. m. morsitans*. Data presented as the mean  $\pm$  S.E.M from three experiments. Legend: MG= midgut infection rate; TI= transmission of infectivity.**

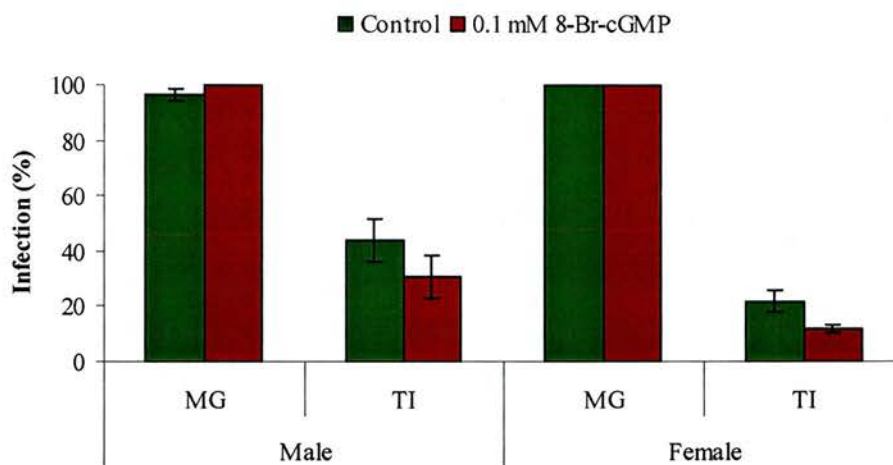
Flies dissected after 28 d showed no significant differences in midgut infections rates in either male or female *G. m. morsitans* with the continual addition of 100  $\mu$ M 8-Br-cGMP. Midgut infection rates in male flies were 96% (n=112) in control flies compared to 92% (n=118) in those continually fed 100  $\mu$ M 8-Br-cGMP ( $\chi^2=1.0$ ,  $p=0.316$ ). Midgut infection rates in female flies were 96% (n=94) in control flies compared to 95% (n=83) in those continually fed 100  $\mu$ M 8-Br-cGMP ( $\chi^2<0.1$ ,  $p=0.858$ ). The continual addition of 100  $\mu$ M 8-Br-cGMP to the bloodmeal had no significant effect on transmission rates in either male or female flies. TI values in male flies were 50% (n=107) in control flies compared to 49% (n=109) in those continually fed 100  $\mu$ M 8-Br-cGMP ( $\chi^2<0.1$ ,  $p=0.896$ ). TI values in female flies were 16% (n=90) in control flies compared to 24% (n=79) in those continually fed 100  $\mu$ M 8-Br-cGMP ( $\chi^2=1.9$ ,  $p=0.166$ ).

There were no significant differences in either midgut infection rates or transmission rates in male flies dissected 14 d post-infection. Midgut infection rates were 97% (n=60) in control flies compared to 90% (n=61) in those continually fed 100  $\mu$ M 8-Br-cGMP ( $\chi^2=2.1$ ,  $p=0.150$ ). TI values were 2% in both control flies (n=58) and those continually fed 100  $\mu$ M 8-Br-cGMP ( $\chi^2<0.1$ ,  $p=0.975$ ).

#### 5.2.4.2 Infective bloodmeal containing GSH

See Section 2.24.3 for method.

The effects of the subsequent addition of 100  $\mu$ M 8-Br-cGMP to the bloodmeal on midgut infection rates and transmission rates of *T. b. brucei* BUT 135 in *G. m. morsitans* when the infective feed was supplemented with 15 mM GSH are shown in Figure 5.6.



**Figure 5.6:** Effects of the subsequent addition of 100  $\mu$ M 8-Br-cGMP to the bloodmeal on midgut infection rates and transmission rates of *T. b. brucei* in *G. m. morsitans* when the infective feed contained 15 mM GSH. Data presented as the mean  $\pm$  S.E.M from three experiments. Legend: MG= midgut infection rate; TI= transmission of infectivity.

To enhance midgut infections to a level at which changes in rate of maturation would be apparent all tsetse flies received an infective bloodmeal supplemented with 15 mM GSH.

Flies dissected after 28 d showed no significant differences in midgut infections rates in either male or female *G. m. morsitans* with the subsequent addition of 100  $\mu$ M 8-Br-cGMP. Midgut infection rates in male flies were 97% (n=87) in control flies compared

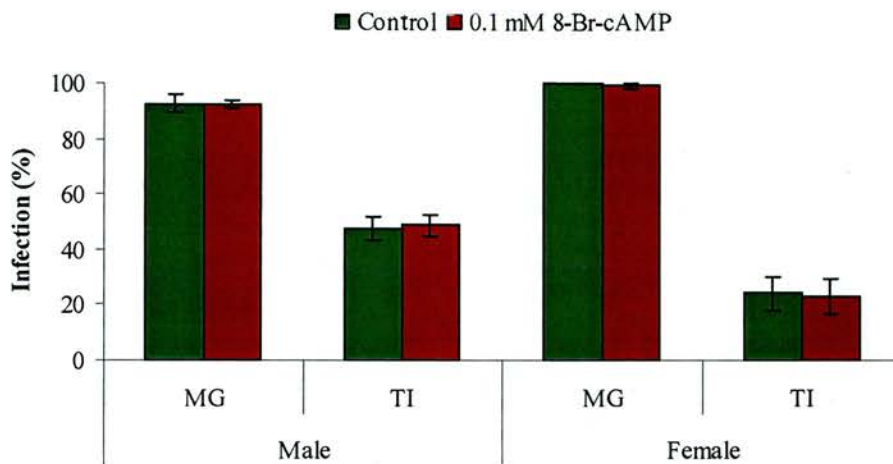
to 100% (n=55) in those subsequently fed 100  $\mu$ M 8-Br-cGMP ( $\chi^2=1.9$ ,  $p=0.164$ ). Midgut infection rates in female flies were 100% in both control flies (n=91) and those subsequently fed 100  $\mu$ M 8-Br-cGMP (n=77). The subsequent addition of 100  $\mu$ M 8-Br-cGMP to the bloodmeal had no significant effect on transmission rates in either male or female flies. TI values in male flies were 44% (n=84) in control flies compared to 31% (n=55) in those continually fed 100  $\mu$ M 8-Br-cGMP ( $\chi^2=2.4$ ,  $p=0.120$ ). TI values in female flies were 22% (n=91) in control flies compared to 12% (n=77) in those continually fed 100  $\mu$ M 8-Br-cGMP ( $\chi^2=3.1$ ,  $p=0.079$ ). Dissection of male flies after 14 d showed no difference in midgut infections rates, however, significant differences were found between transmission rates. Midgut infections rates were both 100%. TI values were 31% (n=74) in control flies compared to 2% (n=60) in those subsequently fed 100  $\mu$ M 8-Br-cGMP ( $\chi^2=19.5$ ,  $p<0.001$ ).

### **5.2.5 The effects of continually feeding 8-Br-cAMP on transmission rates of *T. b. brucei* in *G. m. morsitans***

#### **5.2.5.1 Infective bloodmeal containing GSH**

See Section 2.24.3 for method.

The effects of the subsequent addition of 100  $\mu$ M 8-Br-cAMP to the bloodmeal on midgut infection rates and transmission rates of *T. b. brucei* BUT 135 in *G. m. morsitans* when the infective feed was supplemented with 15 mM GSH are shown in Figure 5.7.



**Figure 5.7:** Effects of the subsequent addition of 100  $\mu$ M 8-Br-cAMP to the bloodmeal on midgut infection rates and transmission rates of *T. b. brucei* in *G. m. morsitans* when the infective feed contained 15 mM GSH. Data presented as the mean  $\pm$  S.E.M from three experiments. Legend; MG= midgut infection rate; TI= transmission of infectivity.

To enhance midgut infections to a level at which changes in rate of maturation would be apparent all tsetse flies received an infective bloodmeal supplemented with 15 mM GSH.

Flies dissected after 28 d showed no significant differences in midgut infection rates in either male or female *G. m. morsitans* with the subsequent addition of 100  $\mu$ M 8-Br-cAMP. Midgut infection rates in male flies were 92% in both control flies ( $n=172$ ) and those subsequently fed 100  $\mu$ M 8-Br-cAMP ( $n=173$ ;  $\chi^2<0.1$ ,  $p=1$ ). Midgut infection rates in female flies were 100% ( $n=95$ ) in control flies compared to 99% ( $n=105$ ) in those subsequently fed 100  $\mu$ M 8-Br-cAMP ( $\chi^2=0.9$ ,  $p=0.343$ ). The subsequent addition of 100  $\mu$ M 8-Br-cAMP to the bloodmeal had no significant effect on transmission rates in either male or female flies. TI values in male flies were 48% ( $n=159$ ) in control flies compared to 49% ( $n=160$ ) in those subsequently fed 100  $\mu$ M 8-Br-cAMP ( $\chi^2<0.1$ ,  $p=0.865$ ). TI values in female flies were 24% ( $n=95$ ) in control flies compared to 23% ( $n=104$ ) in those subsequently fed 100  $\mu$ M 8-Br-cAMP ( $\chi^2<0.1$ ,  $p=0.852$ ).

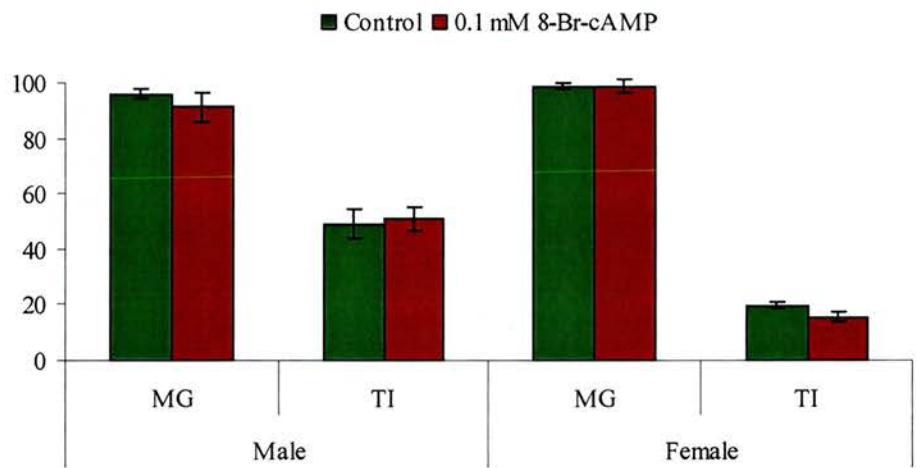


Dissection of male flies after 14 d showed no significant differences in either midgut infection rates or transmission rates. Midgut infections rates were both 100% while TI values were 31% (n=74) in control flies and 30% (n=54) in those subsequently fed 100  $\mu$ M 8-Br-cAMP ( $\chi^2<0.1$ ,  $p=0.860$ ).

### 5.2.5.2 Infective bloodmeal containing 8-Br-cGMP

See Section 2.24.2 for method.

The effects of the subsequent addition of 100  $\mu$ M 8-Br-cAMP on midgut infection rates and transmission rates of *T. b. brucei* BUT 135 in *G. m. morsitans* when the infective feed was supplemented with 100  $\mu$ M 8-Br-cGMP are shown in Figure 5.8.



**Figure 5.8:** Effects of the subsequent addition of 100  $\mu$ M 8-Br-cAMP to the bloodmeal on midgut infection rates and transmission rates of *T. b. brucei* in *G. m. morsitans* when the infective feed contained 100  $\mu$ M 8-Br-cGMP. Data presented as the mean  $\pm$  S.E.M from three experiments. Legend; MG= midgut infection rate; TI= transmission of infectivity.

To enhance midgut infections to a level at which changes in rate of maturation would be apparent all tsetse flies received an infective bloodmeal supplemented with 100  $\mu$ M 8-Br-cGMP.

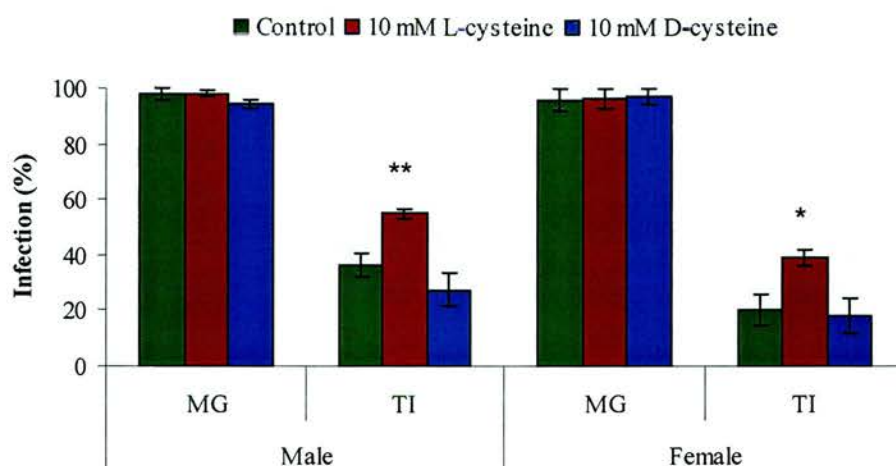
Flies dissected after 28 d showed no significant differences in midgut infection rates in either male or female *G. m. morsitans* with the subsequent addition of 100  $\mu$ M 8-Br-cAMP. Midgut infection rates in male flies were 96% (n=74) in control flies compared to 91% (n=58) in those subsequently fed 100  $\mu$ M 8-Br-cAMP ( $\chi^2=1.2$ ,  $p=0.275$ ). Midgut infection rates in female flies were 99% in both control flies (n=87) and those subsequently fed 100  $\mu$ M 8-Br-cAMP (n=90;  $\chi^2<0.1$ ,  $p=0.975$ ). The subsequent addition of 100  $\mu$ M 8-Br-cAMP to the bloodmeal had no significant effect on transmission rates in either male or female flies. TI values in male flies were 49% (n=71) in control flies compared to 51% (n=53) in those subsequently fed 100  $\mu$ M 8-Br-cAMP ( $\chi^2<0.1$ ,  $p=0.856$ ). TI values in female flies were 20% (n=86) in control flies compared to 16% (n=89) in those subsequently fed 100  $\mu$ M 8-Br-cAMP ( $\chi^2=0.5$ ,  $p=0.484$ ).

#### **5.2.6 The effects of continually feeding cysteine on transmission rates of *T. b. brucei* in *G. m. morsitans***

See Section 2.24.2 for method.

The effects of the subsequent addition of 10 mM L- or D-cysteine to the bloodmeal on midgut infection rates and transmission rates of *T. b. brucei* BUT 135 in *G. m. morsitans* when the infective feed was supplemented with 100  $\mu$ M 8-Br-cGMP are shown in Figure 5.9.





**Figure 5.9: Effects of the subsequent addition of 10 mM L-or D-cysteine to the bloodmeal on midgut infection rates and transmission rates of *T. b. brucei* in *G. m. morsitans* when the infective feed contained 100  $\mu$ M 8-Br-cGMP. Data presented as the mean  $\pm$  S.E.M from three experiments. Legend; MG= midgut infection rate; TI= transmission of infectivity.  $\chi^2$  tests: \*\*  $p<0.01$ ; \*  $p<0.05$  versus the corresponding control value.**

To enhance midgut infections to a level at which changes in rate of maturation would be apparent all tsetse flies received an infective bloodmeal supplemented with 100  $\mu$ M 8-Br-cGMP.

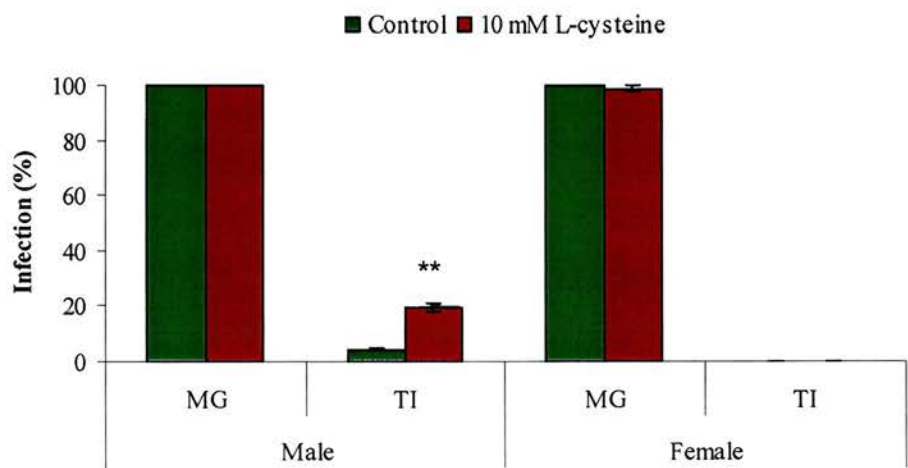
Flies dissected after 28 d showed no significant differences in midgut infection rates in either male or female *G. m. morsitans* with the subsequent addition of either 10 mM L- or D-cysteine when compared with controls which received no cysteine. Midgut infection rates in male flies were 98% (n=106) in control flies compared to 98% (n=113) in those subsequently fed L-cysteine and 94% (n=85) in those subsequently fed D-cysteine (compared with controls: L-cysteine:  $\chi^2<0.1$ ,  $p=0.949$ ; D-cysteine:  $\chi^2=2.1$ ,  $p=0.144$ ). Midgut infection rates in female flies were 96% (n=72) in control flies compared to 96% (n=85) in those subsequently fed L-cysteine and 97% (n=68) in those subsequently fed D-cysteine (compared with controls: L-cysteine:  $\chi^2<0.1$ ,  $p=0.836$ ; D-cysteine:  $\chi^2=0.2$ ,  $p=0.696$ ). L-cysteine significantly increased the proportions of midgut infections maturing into salivary gland infections from a control value of 37% (n=104)

to 55% (n=111) in males and from a control value of 20% (n=69) to 39% (n=82) in females (male:  $\chi^2=7.3$ ,  $p=0.007$ ; female:  $\chi^2=6.2$ ,  $p=0.013$ ). The addition of D-cysteine resulted in TI values of 28% (n=80) in males and 18% (n=66) in females which were not significantly different from controls (males:  $\chi^2=1.7$ ,  $p=0.195$ ; females:  $\chi^2<0.1$ ,  $p=0.876$ ). Differences in TI between the addition of L- or D-cysteine were significant in both male and females (male:  $\chi^2=14.3$ ,  $p<0.001$ ; female:  $\chi^2=7.6$ ,  $p=0.006$ ).

### 5.2.7 The effects of continually feeding cysteine on transmission rates of *T. b. rhodesiense* in *G. m. morsitans*

See Section 2.25.1 for method.

The effects of the subsequent addition of 10 mM L-cysteine to the bloodmeal on midgut infection rates and transmission rates of *T. b. rhodesiense* DO in *G. m. morsitans* when the infective feed was supplemented with 100  $\mu$ M 8-Br-cGMP are shown in Figure 5.10.



**Figure 5.10:** Effects of the subsequent addition of 10 mM L-cysteine to the bloodmeal on midgut infection rates and transmission rates of *T. b. rhodesiense* in *G. m. morsitans* when the infective feed contained 100  $\mu$ M 8-Br-cGMP. Data presented as the mean  $\pm$  S.E.M from three experiments. Legend; MG= midgut infection rate; TI= transmission of infectivity.  $\chi^2$  tests: \*\*  $p<0.01$  versus the corresponding control value.

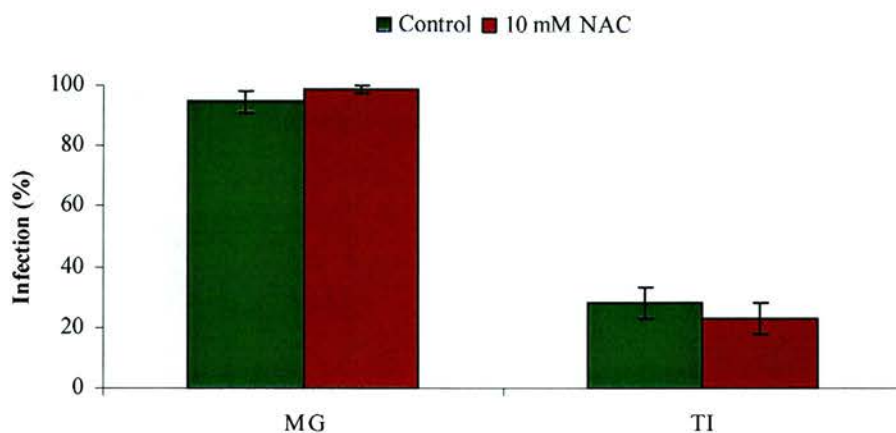
To enhance midgut infections to a level at which changes in rate of maturation would be apparent all tsetse flies received an infective bloodmeal supplemented with 100  $\mu$ M 8-Br-cGMP.

Flies dissected after 28 d showed no significant differences in midgut infection rates in either male or female *G. m. morsitans* with the subsequent addition of 10 mM L-cysteine when compared with controls which received no cysteine. Midgut infection rates in male flies were 100% in both control flies (n=89) and those subsequently fed 10 mM L-cysteine (n=91). Midgut infection rates in female flies were 100% (n=86) in control flies compared to 99% (n=89) in those subsequently fed 10 mM L-cysteine ( $\chi^2=1.0$ ,  $p=0.308$ ). The subsequent addition of 10 mM L-cysteine significantly increased transmission in male flies, however, there were no significant differences in transmission rates in female flies. TI values in male flies were increased from 4% (n=89) in control flies to 20% (n=91) in those subsequently fed 10 mM L-cysteine ( $\chi^2=9.8$ ,  $p=0.002$ ). No mature infections were observed in female flies in either the control group (n=89) or those subsequently fed 10 mM L-cysteine (n=85).

#### **5.2.8 The effects of continually feeding NAC on transmission rates of *T. b. brucei* in *G. m. morsitans***

See Section 2.24.2 for method.

The effects of the subsequent addition of 10 mM NAC to the bloodmeal on midgut infection rates and transmission rates of *T. b. brucei* BUT 135 in *G. m. morsitans* when the infective feed was supplemented with 100  $\mu$ M 8-Br-cGMP are shown in Figure 5.11.



**Figure 5.11: Effects of the subsequent addition of 10 mM NAC to the bloodmeal on midgut infection rates and transmission rates of *T. b. brucei* in *G. m. morsitans* when the infective feed contained 100  $\mu$ M 8-Br-cGMP. Data presented as the mean  $\pm$  S.E.M from three experiments. Legend; MG= midgut infection rate; TI= transmission of infectivity.**

To enhance midgut infections to a level at which changes in rate of maturation would be apparent all tsetse flies received an infective bloodmeal supplemented with 100  $\mu$ M 8-Br-cGMP.

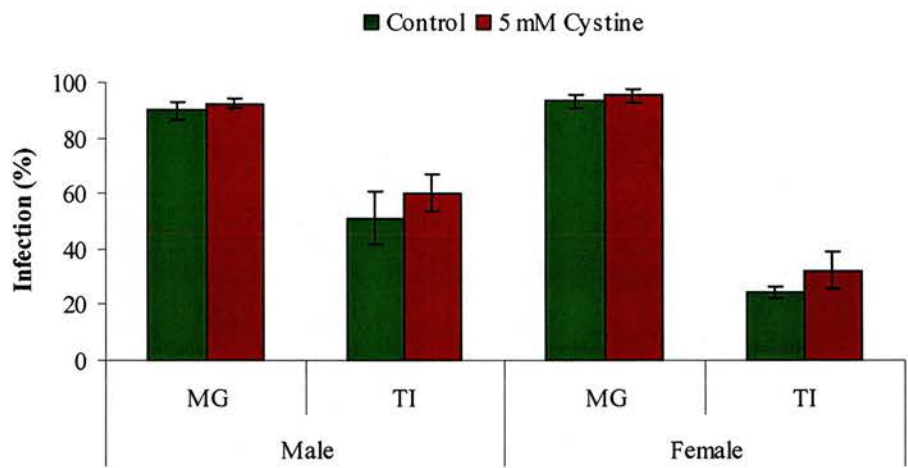
Flies dissected after 28 d showed no significant differences in midgut infection rates in male *G. m. morsitans* with the subsequent addition of 10 mM NAC. Midgut infection rates in male flies were 95% (n=93) in control flies compared to 99% (n=88) in those subsequently fed 10 mM NAC ( $\chi^2=2.5$ ,  $p=0.111$ ). The subsequent addition of 10 mM NAC had no significant effect on transmission in male flies. TI values were 28% (n=88) in control flies compared to 23% (n=87) in those subsequently fed 10 mM NAC ( $\chi^2=0.7$ ,  $p=0.412$ ).



**5.2.9 The effects of continually feeding cystine on transmission rates of *T. b. brucei* in *G. m. morsitans***

See Section 2.24.2 for method.

The effects of the subsequent addition of 5 mM cystine to the bloodmeal on midgut infection rates and transmission rates of *T. b. brucei* BUT 135 in *G. m. morsitans* when the infective feed was supplemented with 100  $\mu$ M 8-Br-cGMP are shown in Figure 5.12.



**Figure 5.12:** Effects of the subsequent addition of 5 mM cystine to the bloodmeal on midgut infection rates and transmission rates of *T. b. brucei* in *G. m. morsitans* when the infective feed contained 100  $\mu$ M 8-Br-cGMP. Data presented as the mean  $\pm$  S.E.M from three experiments. Legend; MG= midgut infection rate; TI= transmission of infectivity.

To enhance midgut infections to a level at which changes in rate of maturation would be apparent all tsetse flies received an infective bloodmeal supplemented with 100  $\mu$ M 8-Br-cGMP.

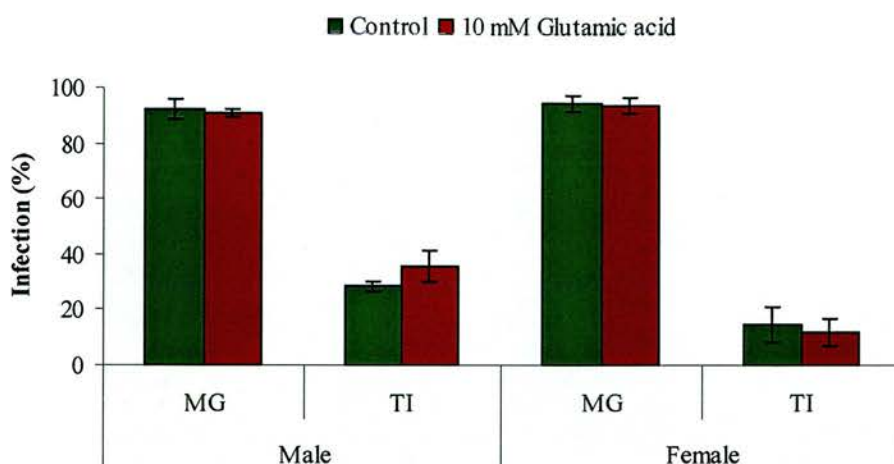
Flies dissected after 28 d showed no significant differences in midgut infection rates in either male or female *G. m. morsitans* with the subsequent addition of 5 mM cystine.

Midgut infection rates in male flies were 90% (n=89) in control flies compared to 93% (n=95) in those subsequently fed 5 mM cystine ( $\chi^2=0.2$ ,  $p=0.644$ ). Midgut infection rates in female flies were 93% (n=92) in control flies compared to 96% (n=94) in those subsequently fed 5 mM cystine ( $\chi^2=1.1$ ,  $p=0.290$ ). The subsequent addition of 5 mM cystine had no significant effect on transmission rates in either male or females. TI values in male flies were 51% (n=80) in control flies compared to 60% (n=88) in those subsequently fed 5 mM cystine ( $\chi^2=3.0$ ,  $p=0.082$ ). TI values in female flies were 24% (n=86) in control flies compared to 32% (n=90) in those subsequently fed 5 mM cystine ( $\chi^2<0.1$ ,  $p=0.896$ )

#### **5.2.10 The effects of continually feeding glutamic acid on transmission rates of *T. b. brucei* in *G. m. morsitans***

See Section 2.24.2 for method.

The effects of the subsequent addition of 10 mM glutamic acid to the bloodmeal on midgut infection rates and transmission rates of *T. b. brucei* BUT 135 in *G. m. morsitans* when flies were infected with 100  $\mu$ M 8-Br-cGMP are shown in Figure 5.13.



**Figure 5.13: Effects of the subsequent addition of 10 mM glutamic acid to the bloodmeal on midgut infection rates and transmission rates of *T. b. brucei* in *G. m. morsitans* when the infective feed contained 100  $\mu$ M 8-Br-cGMP. Data presented as the mean  $\pm$  S.E.M from three experiments. Legend; MG= midgut infection rate; TI= transmission of infectivity.**

To enhance midgut infections to a level at which changes in rate of maturation would be apparent all tsetse flies received an infective bloodmeal supplemented with 100  $\mu$ M 8-Br-cGMP.

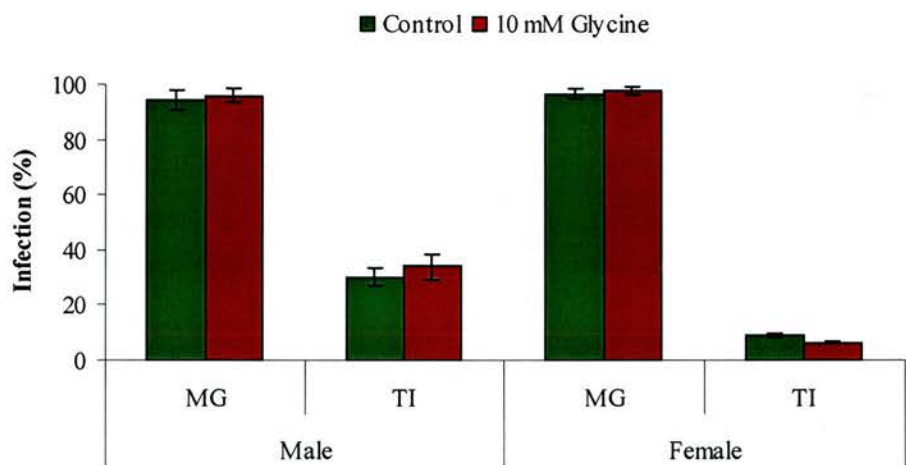
Flies dissected after 28 d showed no significant differences in midgut infection rates in either male or female *G. m. morsitans* with the subsequent addition of 10 mM glutamic acid. Midgut infection rates in male flies were 92% (n=91) in control flies compared to 91% (n=99) in those subsequently fed 10 mM glutamic acid ( $\chi^2=0.1$ ,  $p=0.729$ ). Midgut infection rates in female flies were 95% (n=93) in control flies compared to 94% (n=98) in those fed 10 mM glutamic acid ( $\chi^2<0.1$ ,  $p=0.825$ ). The subsequent addition of 10 mM glutamic acid had no significant effect on transmission rates in either male or females. TI values in male flies were 29% (n=84) in control flies compared to 36% (n=90) in those subsequently fed 10 mM glutamic acid ( $\chi^2=1.0$ ,  $p=0.324$ ). TI values in female flies were 15% (n=88) in control flies compared to 12% (n=92) in those subsequently fed 10 mM glutamic acid (n=90;  $\chi^2=0.3$ ,  $p=0.578$ ).



**5.2.11 The effects of continually feeding glycine on transmission rates of *T. b. brucei* in *G. m. morsitans***

See Section 2.24.2 for method.

The effects of the subsequent addition of 10 mM glycine on midgut infection rates and transmission rates of *T. b. brucei* BUT 135 in *G. m. morsitans* when the infective feed was supplemented with 100  $\mu$ M 8-Br-cGMP are shown in Figure 5.14.



**Figure 5.14:** Effects of the subsequent addition of 10 mM glycine to the bloodmeal on midgut infection rates and transmission rates of *T. b. brucei* in *G. m. morsitans* when the infective feed contained 100  $\mu$ M 8-Br-cGMP. Data presented as the mean  $\pm$  S.E.M from three experiments. Legend; MG= midgut infection rate; TI= transmission of infectivity.

To enhance midgut infections to a level at which changes in rate of maturation would be apparent all tsetse flies received an infective bloodmeal supplemented with 100  $\mu$ M 8-Br-cGMP.

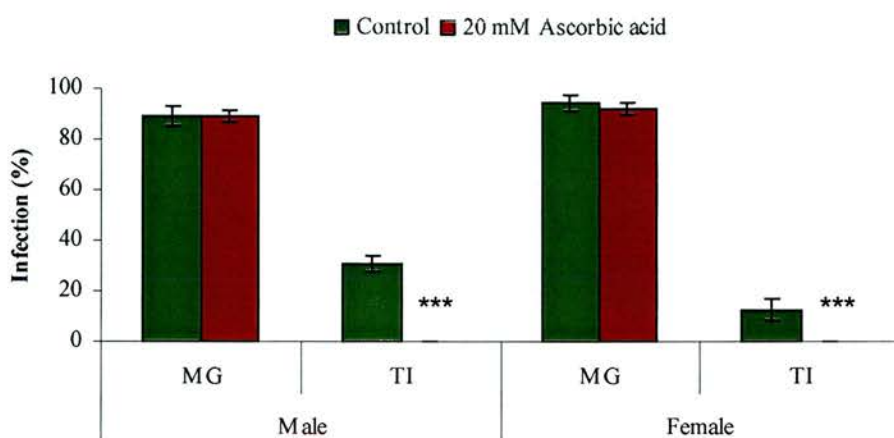
Flies dissected after 28 d showed no significant differences in midgut infection in either male or female *G. m. morsitans* with the subsequent addition of 10 mM glycine. Midgut infection rates in male flies were 95% (n=91) in control flies compared to 96% (n=98) in those subsequently fed 10 mM glycine ( $\chi^2=0.2$ ,  $p=0.648$ ). Midgut infection rates in

female flies were 97% (n=86) in control flies compared to 98% (n=94) in those subsequently fed 10 mM glycine ( $\chi^2=0.2$ ,  $p=0.633$ ). The subsequent addition of 10 mM glycine had no significant effect on transmission in either male or females. TI values in male flies were 30% (n=86) in control flies compared to 34% (n=94) in those subsequently fed 10 mM glycine ( $\chi^2=0.3$ ,  $p=0.585$ ). TI values in female flies were 9% (n=90) in control flies compared to 6% (n=93) in those subsequently fed 10 mM glycine ( $\chi^2=0.4$ ,  $p=0.535$ ).

#### **5.2.12 The effects of continually feeding ascorbic acid on transmission rates of *T. b. brucei* in *G. m. morsitans***

See Section 2.24.2 for method.

The effects of the subsequent addition of 20 mM ascorbic acid on midgut infection rates and transmission rates of *T. b. brucei* BUT 135 in *G. m. morsitans* when the infective feed was supplemented with 100  $\mu$ M 8-Br-cGMP are shown in Figure 5.15.



**Figure 5.15: Effects of the subsequent addition of 20 mM ascorbic acid to the bloodmeal on midgut infection rates and transmission rates of *T. b. brucei* in *G. m. morsitans* when the infective feed contained 100  $\mu$ M 8-Br-cGMP. Data presented as the mean  $\pm$  S.E.M from three experiments. Legend; MG= midgut infection rate; TI= transmission of infectivity.  $\chi^2$  tests: \*\*\*  $p < 0.001$  versus the corresponding control value.**

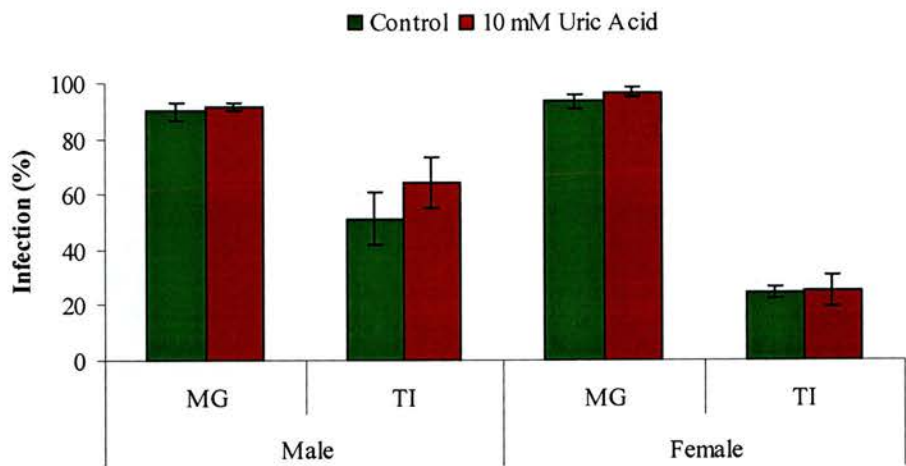
To enhance midgut infections to a level at which changes in rate of maturation would be apparent all tsetse flies received an infective bloodmeal supplemented with 100  $\mu$ M 8-Br-cGMP.

Flies dissected after 28 d showed no significant differences in midgut infection rates in either male or female *G. m. morsitans* with the subsequent addition of 20 mM ascorbic acid. Midgut infection rates in male flies were 89% in both controls (n=92) and those subsequently fed 20 mM ascorbic acid (n=83;  $\chi^2 < 0.1$ ,  $p > 0.999$ ). Midgut infection rates in female flies were 95% (n=92) in control flies compared to 92% (n=92) in those subsequently fed 20 mM ascorbic acid ( $\chi^2 = 0.4$ ,  $p = 0.550$ ). Ascorbic acid completely blocked maturation in both males and females, reducing TI from 30% (n=82) to 0% (n=74) in males and from 13% (n=87) to 0% (n=85) in females (male:  $\chi^2 = 26.9$ ,  $p < 0.001$ ; female:  $\chi^2 = 11.5$ ,  $p < 0.001$ ). The subsequent addition of 1 mM ascorbic acid had no significant effect on transmission rates when flies were infected with 100  $\mu$ M 8-Br-cGMP. TI values were 44% (n=27) in control flies compared to 42% (n=19) in those subsequently fed 1 mM ascorbic acid ( $\chi^2 = 0.1$ ,  $p = 0.760$ ).

**5.2.13 The effects of continually feeding uric acid on transmission rates of *T. b. brucei* in *G. m. morsitans***

See Section 2.24.2 for method.

The effects of the subsequent addition of uric acid on midgut infection rates and transmission rates of *T. b. brucei* BUT 135 in *G. m. morsitans* when the infective feed was supplemented with 100  $\mu$ M 8-Br-cGMP are shown in Figure 5.16.



**Figure 5.16: Effects of the subsequent addition of 20 mM uric acid to the bloodmeal on midgut infection rates and transmission rates of *T. b. brucei* in *G. m. morsitans* when the infective feed contained 100  $\mu$ M 8-Br-cGMP. Data presented as the mean  $\pm$  S.E.M from three experiments. Legend; MG= midgut infection rate; TI= transmission of infectivity.**

To enhance midgut infections to a level at which changes in rate of maturation would be apparent all tsetse flies received an infective bloodmeal supplemented with 100  $\mu$ M 8-Br-cGMP.

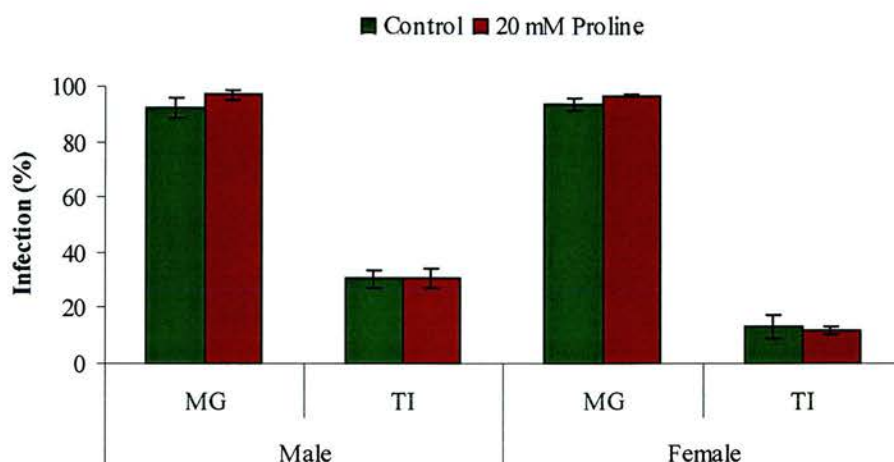
Flies dissected after 28 d showed no significant differences in midgut infections rates in either male or female *G. m. morsitans* with the subsequent addition of 10 mM uric acid. Midgut infection rates in male flies were 90% (n=89) in control flies compared to 92% (n=98) in those fed 10 mM uric acid ( $\chi^2=0.2$ ,  $p=0.644$ ). Midgut infection rates in female flies were 93% (n=92) in control flies compared to 97% (n=94) in those fed 10 mM uric acid ( $\chi^2=1.1$ ,  $p=0.290$ ). The subsequent addition of 10 mM uric acid had no significant effect on transmission in either male or female flies. TI values in male flies were 51% (n=80) in control flies compared to 64% (n=90) in those continually fed 10 mM uric acid ( $\chi^2=3.0$ ,  $p=0.082$ ). TI values in female flies were 24% (n=86) in control flies compared to 25% (n=91) in those subsequently fed 10 mM uric acid ( $\chi^2<0.1$ ,  $p=0.896$ ).

#### **5.2.14 The effects of continually feeding proline on transmission rates of *T. b. brucei* in *G. m. morsitans***

See Section 2.24.2 for method.

The effects of the subsequent addition of 10 mM proline on midgut infection rates and transmission rates of *T. b. brucei* BUT 135 in *G. m. morsitans* when the infective feed was supplemented with 100  $\mu$ M 8-Br-cGMP are shown in Figure 5.17.





**Figure 5.17:** Effects of the subsequent addition of 20 mM proline to the bloodmeal on midgut infection rates and transmission rates of *T. b. brucei* in *G. m. morsitans* when the infective feed contained 100  $\mu$ M 8-Br-cGMP. Data presented as the mean  $\pm$  S.E.M from three experiments. Legend; MG= midgut infection rate; TI= transmission of infectivity.

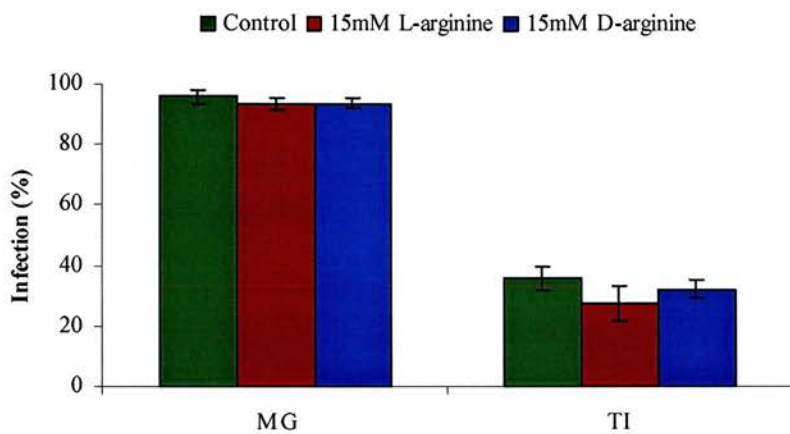
To enhance midgut infections to a level at which changes in rate of maturation would be apparent all tsetse flies received an infective bloodmeal supplemented with 100  $\mu$ M 8-Br-cGMP.

Flies dissected after 28 d showed no significant differences in midgut infections in either male or female *G. m. morsitans* with the subsequent addition of 20 mM proline. Midgut infection rates in male flies were 92% (n=92) in control flies compared to 97% (n=101) in those subsequently fed 20 mM proline ( $\chi^2=2.1$ ,  $p=0.147$ ). Midgut infection rates in female flies were 94% (n=92) in control flies compared to 97% (n=94) in those subsequently fed 10 mM proline ( $\chi^2=1.0$ ,  $p=0.321$ ). The subsequent addition of 20 mM proline had no significant effect on transmission rates in either male or females flies. TI values in male flies were 31% in both control flies (n=85) and those subsequently fed 20 mM proline (n=98;  $\chi^2<0.1$ ,  $p>0.999$ ). TI values in female flies were 13% (n=91) in control flies compared to 12% (n=92) in those subsequently fed 20 mM proline ( $\chi^2<0.1$ ,  $p=0.802$ ).

### 5.2.15 The effects of continually feeding arginine on transmission rates of *T. b. brucei* in *G. m. morsitans*

See Section 2.24.2 for method.

The effects of the subsequent addition of L- or D-arginine on midgut infection rates and transmission rates of *T. b. brucei* BUT 135 in male *G. m. morsitans* when the infective feed was supplemented with 100  $\mu$ M 8-Br-cGMP are shown in Figure 5.18.



**Figure 5.18:** Effects of the subsequent addition of 15 mM L- or D-arginine to the bloodmeal on midgut infection rates and transmission rates of *T. b. brucei* in *G. m. morsitans* when the infective feed contained 100  $\mu$ M 8-Br-cGMP. Data presented as the mean  $\pm$  S.E.M from three experiments. Legend; MG= midgut infection rate; TI= transmission of infectivity.

To enhance midgut infections to a level at which changes in rate of maturation would be apparent all tsetse flies received an infective bloodmeal supplemented with 100  $\mu$ M 8-Br-cGMP.

Flies dissected after 28 d showed no significant differences in midgut infection rates in male *G. m. morsitans* with the subsequent addition of either 15 mM L- or D-arginine. Midgut infection rates in male flies were 97% (n=99) in control flies compared to 94% in flies fed either L- or D-arginine (L-arginine: n=108;  $\chi^2=0.6$ ,  $p=0.434$ ; D-arginine:

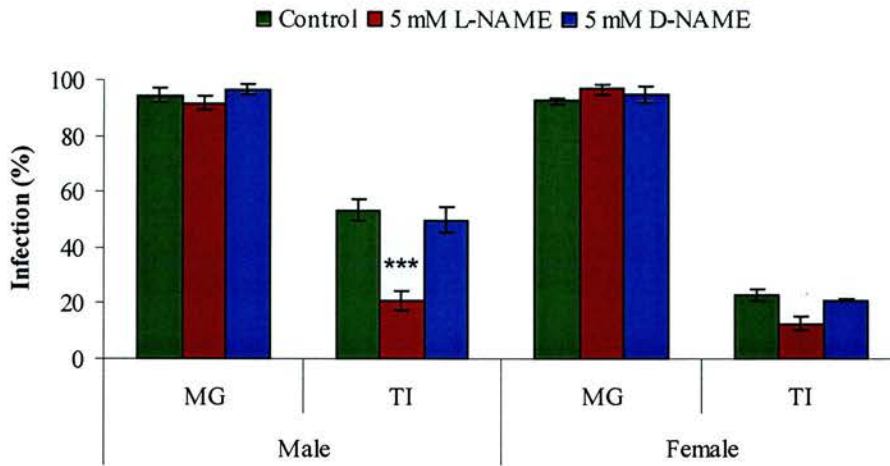


n=96;  $\chi^2=0.5$ ,  $p=0.484$ ). The subsequent addition of 15 mM L-or D-arginine had no significant effect on transmission rates in male flies. TI values in male flies were 36% (n=95) in control flies compared to 28% (n=101) in flies subsequently fed L-arginine and 32% (n=90) in flies subsequently fed D-arginine (L-arginine:  $\chi^2=1.5$ ,  $p=0.225$ ; D-arginine:  $\chi^2=0.3$ ,  $p=0.609$ ). There were no significant differences in transmission rates between the addition of L- or D-arginine ( $\chi^2=0.4$ ,  $p=0.458$ ).

#### **5.2.16 The effects of continually feeding NAME or aminoguanidine on transmission rates of *T. b. brucei* in *G. m. morsitans***

See Section 2.24.2 for method.

The effects of L- or D-NAME on midgut infection rates and transmission rates of *T. b. brucei* BUT 135 in *G. m. morsitans* when the infective feed was supplemented with 100  $\mu$ M 8-Br-cGMP are shown in Figure 5.19.



**Figure 5.19: Effects of the subsequent addition of 5 mM L- or D-NAME to the bloodmeal on midgut infection rates and transmission rates of *T. b. brucei* in *G. m. morsitans* when the infective feed contained 100  $\mu$ M 8-Br-cGMP. Data presented as the mean  $\pm$  S.E.M from three experiments. Legend; MG= midgut infection rate; TI= transmission of infectivity.  $\chi^2$  tests: \*\*\*  $p < 0.001$  versus the corresponding control value.**

To enhance midgut infections to a level at which changes in rate of maturation would be apparent all tsetse flies received an infective bloodmeal supplemented with 100  $\mu$ M 8-Br-cGMP.

Flies dissected after 28 d showed no significant differences in midgut infections rates in either male or female *G. m. morsitans* with the subsequent addition of either 5 mM L- or D-NAME when compared with controls which received no NAME. Midgut infection rates in male flies were 95% (n=91) in control flies compared to 92% (n=98) in those subsequently fed L-NAME and 97% (n=95) in those subsequently fed D-NAME (compared to controls: L-NAME:  $\chi^2=0.5$ ,  $p=0.469$ ; D-NAME:  $\chi^2=0.6$ ,  $p=0.432$ ). Midgut infection rates in female flies were 93% (n=99) in control flies compared to 97% (n=96) in those subsequently fed L-NAME and 95% (n=99) in those subsequently fed D-NAME (compared to controls: L-NAME:  $\chi^2=1.6$ ,  $p=0.206$ ; D-NAME:  $\chi^2=0.4$ ,  $p=0.539$ ). L-NAME significantly reduced TI values from a control value of 53% (n=86) to 21% (n=90) in male flies ( $\chi^2=19.8$ ,  $p < 0.001$ ). Although TI values were decreased from 23%

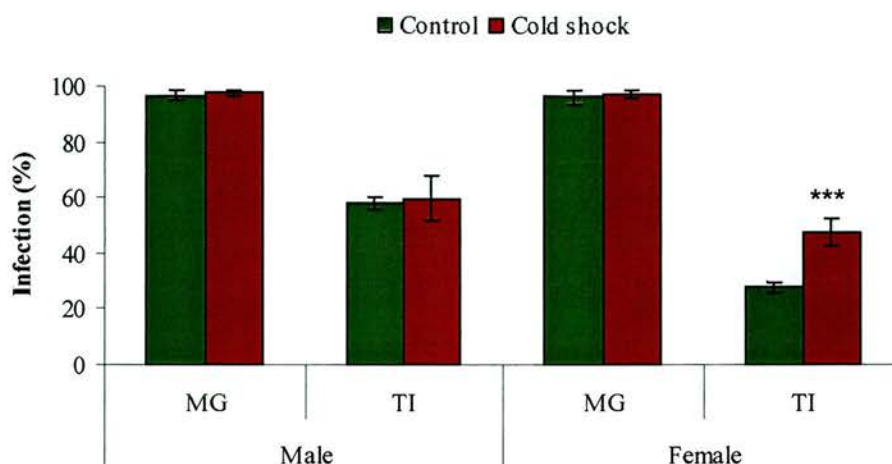
(n=96) to 13% (n=93) in females, the difference was not significant ( $\chi^2=3.2$ ,  $p=0.072$ ). The addition of D-NAME resulted in TI values of 50% (n=92) in males and 21% (n=94) in females which were not significantly different from controls (males:  $\chi^2=0.2$ ,  $p=0.641$ ; females:  $\chi^2=0.4$ ,  $p=0.539$ ). Differences in TI rates between the addition of L- or D-NAME were significant in males but not in females (males:  $\chi^2=16.5$ ,  $p<0.001$ ; females:  $\chi^2=2.3$ ,  $p=0.128$ ).

The subsequent addition of 15 mM aminoguanidine after the infective bloodmeal which was supplemented with 100  $\mu$ M 8-Br-cGMP significantly reduced both the number of midgut infections and transmission rates in male tsetse. Midgut infections were reduced from a control value of 92% (n=104) to 52% (n=99;  $\chi^2=42.2$ ,  $p<0.001$ ). TI values were reduced from 64% (n=96) to 0% (n=51;  $\chi^2=55.4$ ,  $p<0.001$ ).

#### **5.2.17 The effects of cold shock four days post-infection on transmission rates of *T. b. brucei* or *T. b. rhodesiense* in *G. m. morsitans***

See Sections 2.24.4 and 2.25.2 for methods.

The effects of cold shock 4 d post-infection on midgut infection rates and transmission rates of *T. b. brucei* BUT 135 in *G. m. morsitans* when the infective feed was supplemented with 15 mM GSH are shown in Figure 5.20.



**Figure 5.20: Effects of cold shock 4 d post-infection on midgut infection rates and transmission rates of *T. b. brucei* in *G. m. morsitans* when the infective feed contained 15 mM GSH. Data presented as the mean  $\pm$  S.E.M from four experiments. Legend; MG= midgut infection rate; TI= transmission of infectivity.  $\chi^2$  tests: \*\*\*  $p < 0.001$  versus the corresponding control value.**

To enhance midgut infections of But 135 to a level at which changes in rate of maturation would be apparent all tsetse flies received an infective bloodmeal supplemented with 15 mM GSH.

Flies dissected after 28 d showed no significant differences in midgut infections rates in either male or female *G. m. morsitans* that were cold shocked for 30 min at 4°C when compared with controls which received no cold shock. Midgut infections in male flies were 97% (n=99) in control flies compared to 98% (n=97) in flies that had been chilled ( $\chi^2=0.1$ ,  $p=0.744$ ). Midgut infections in female flies were 96% (n=131) in control flies compared to 97% (n=147) in flies that had been chilled ( $\chi^2=0.3$ ,  $p=0.607$ ). Cold shock significantly increased the proportions of midgut infections maturing into salivary gland infections in female tsetse from a control value of 28% (n=126) to 48% (n=143;  $\chi^2=11.1$ ,  $p < 0.001$ ). Cold shock had no significant effect on proportions of midgut infections maturing into salivary gland infections in male tsetse, TI values were 58% in control flies compared to 60% in flies which been chilled ( $\chi^2 < 0.1$ ,  $p=0.840$ ).

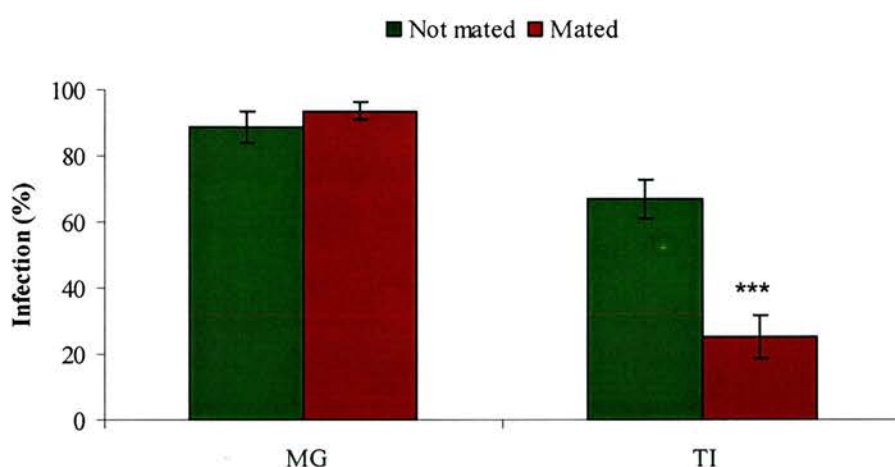
Dissection of flies after 21 d resulted in significant rises in transmission rates from a control value of 11% (n=28) to 39% (n=31) and 34% (n=29) in flies chilled for 5 min and 1 h respectively (5 m:  $\chi^2=6.1$ ,  $p=0.014$ ; 1h:  $\chi^2=4.6$ ,  $p=0.033$ ). Although chilling for 3 h increased transmission rates to 32% (n=19) the differences were not significant ( $\chi^2=3.2$ ,  $p=0.074$ ). Dissection of flies at 14 d showed no significant differences in transmission rates in either females or males. Female flies which had been chilled (n=27) and control flies (n=28) both produced transmission rates of 7% ( $\chi^2<0.1$ ,  $p=0.975$ ). Male flies showed transmission rates of 19% (n=31) in control flies compared to 7% (n=27) in those that were chilled ( $\chi^2=1.7$ ,  $p=0.188$ ).

No significant differences were found in transmission rates of male or female *G. m. morsitans* when tsetse infected with DO were subjected to cold shock. To enhance midgut infections to a level at which changes in rate of maturation would be apparent all tsetse flies received an infective bloodmeal supplemented with 15 mM GSH. No significant differences were found between the midgut infection rates of male or female flies after cold shock. Midgut infections in male flies were 97% (n=29) in control flies compared to 96% (n=27) in flies that had been chilled ( $\chi^2<0.1$ ,  $p=0.956$ ). Midgut infections rates in female flies were 94% (n=32) in control flies compared to 93% (n=28) in flies that had been chilled ( $\chi^2<0.1$ ,  $p=0.890$ ). TI values in male flies were 7% (n=26) in control flies compared to 8% (n=27) in flies that had been chilled ( $\chi^2<0.1$ ,  $p=0.938$ ). No mature infections were observed in female flies in either the control group (n=30) or those subjected to cold shock (n=26).

### 5.2.18 The effects of mating on transmission rates of *T. b. brucei* in female *G. m. morsitans*

See Section 2.24.5 for method.

The effects of mating on midgut infection rates and transmission rates of *T. b. brucei* BUT 135 in female *G. m. morsitans* when the infective feed was supplemented with 15 mM GSH are shown in Figure 5.21



**Figure 5.21:** Effects of mating on midgut infection rates and transmission rates of *T. b. brucei* in *G. m. morsitans* when the infective feed contained 15 mM GSH. Data presented as the mean  $\pm$  S.E.M from four experiments. Legend; MG= midgut infection rate; TI= transmission of infectivity.  $\chi^2$  tests: \*\*\*  $p < 0.001$  versus the corresponding control value.

To enhance midgut infections to a level at which changes in rate of maturation would be apparent all tsetse flies received an infective bloodmeal supplemented with 15 mM GSH.

Flies dissected after 28 d showed no significant differences in midgut infections rates in female *G. m. morsitans* that were mated compared to unmated controls. Midgut infections rates were 89% (n=99) in unmated flies compared to 94% (n=97) in mated flies ( $\chi^2=1.5$ ,  $p=0.221$ ). However, mating significantly reduced the proportions of



midgut infections maturing into salivary gland infections from a control value of 67% (n=88) to 25% (n=91;  $\chi^2=31.4$ ,  $p<0.001$ ).

### **5.2.19 Re-examination of data from Otieno *et al.* (1983)**

See Section 2.26.8 for details on statistical analyses and Appendix 1 for methods.

Flies that were chilled post-infection 1-8 h after emergence produced midgut infection rates of 41% (n=103) and TI values of 61% (n=42) compared to flies that were not chilled which produced midgut infection rates of 28% (n=99) and TI values of 46% (n=28). Flies that were chilled post-infection 12-24 h after emergence produced midgut infection rates of 24% (n=93) and TI values of 59% (n=22) compared to flies which were not chilled which produced midgut infection rates of 17% (n=84) and TI values of 50% (n=14). If the two time points are added together then the total midgut infections rates were 33% (n=196) in chilled flies compared to 23% (n=183) in control flies, with TI values of 61% (n=64) in chilled flies compared to 48% (n=42) in control flies. Logistic regression analysis of the data showed significant differences between midgut infection rates between those that had been chilled and control flies ( $\chi^2=4.8$ ,  $p=0.029$ ). However, no significant differences were found between the TI values of chilled and control flies ( $\chi^2=1.8$ ,  $p=0.177$ ).



## 5.3 Discussion

To move from one mammalian host to another the trypanosome must undergo cyclical development in the tsetse. Once established in the tsetse gut trypanosomes must move out of the ectoperitrophic space and make their way to the salivary glands, where they can mature into mammalian infective forms.

Previous work indicated that trypanosomes rely on a signal from tsetse lectins to mature (Welburn and Maudlin, 1989). Inhibition of the midgut or haemolymph lectins with the sugars glucosamine and melibiose respectively significantly reduced the number of flies with mature infections (Maudlin and Welburn, 1988; Maudlin and Welburn, 1990).

Removal of serum from the tsetse diet also reduced the proportions of mature infections and it was suggested that  $\gamma$ -globulin and lipids played a role in promoting the maturation of trypanosome infections (Maudlin *et al.*, 1984).

The current work has shown that it is possible to artificially increase the levels of transmission of *T. b. brucei* by two distinct processes.

### 5.3.1 The effects of antioxidants on maturation of trypanosome midgut infections in *G. m. morsitans*

The provision of different antioxidants had contradictory results suggesting the oxidant state of the midgut environment does not play a role in potentiating transmission. The continual addition of L-cysteine resulted in a significant increase in the proportion of midgut infections maturing. The antioxidants GSH, uric acid, D-cysteine and NAC had no significant effects on maturation rates, while the continual addition of the antioxidants ascorbic acid and L-NAME significantly reduced maturation rates.

In the previous chapter it was shown that both L- and D-cysteine promote the same levels of trypanosome midgut infections, suggesting that they detoxify the midgut environment of toxic reactive oxygen species (ROS). However, continuous feeding of

the two isomers produced significant differences in maturation rates, L-cysteine increased transmission while D-cysteine had no effect. The two isomers differ in respect to protein synthesis; L-cysteine can be used in the synthesis of proteins whereas D-cysteine cannot (Glazenburg *et al.*, 1984; Duszenko *et al.*, 1992). Since D-cysteine had no effect on maturation rates it is likely that the increase in maturation of *T. b. brucei* following addition of L-cysteine is due to the production of proteins. The addition of L-cysteine also significantly increased transmission rates in male flies infected with *T. b. rhodesiense*.

Previous studies have shown that bloodstream trypanosomes cannot use cystine and have to be either provided with cysteine or co-cultured with insect cells which can break down cystine to cysteine (Duszenko *et al.*, 1992), while cystine provided in the diet of the mosquito *Aedes aegypti* can be efficiently transformed into GSH (Hazelton and Lang, 1983). In the present work, however, provision of L-cystine had no significant effect on maturation rates and it is likely that the L-cysteine provided in the bloodmeal is being utilised directly by the trypanosome and not by the fly. Although cystine produced an increase in maturation it was not significant and suggests that tsetse cells could release some L-cysteine into the midgut which could be taken up by the trypanosomes. L-cysteine is one of the building blocks of trypanothione the main antioxidant molecule found in trypanosomes and raised levels of trypanothione may be important to the trypanosome to promote its journey to the salivary glands. The addition of glutamic acid or glycine to the infective feed did not significantly affect transmission suggesting that if trypanothione is involved in maturation then the rate limiting molecule is L-cysteine. As well as trypanothione other antioxidant molecules of trypanosomes contain cysteine residues including the active site of trypanothione, which is responsible for detoxifying hydrogen peroxide and organic peroxides (Alphey *et al.*, 2003).

Cysteine has been shown to be important in the lifecycle of *Chlamydia* spp. with the synthesis and insertion into the outer membrane of three cysteine-rich proteins accompanying the differentiation from the reproductive form to the infective form (Allan *et al.*, 1985). The surface coat of procyclic trypanosomes, procyclin, does not contain any cysteine residues and is composed entirely of the amino acids glycine,

proline, glutamic acid and threonine making up amino acid repeats of GPEET and EP; however, a procyclin-associated gene (Koenig-Martin *et al.*, 1992; Roditi *et al.*, 1998) and cysteine-rich repetitive acidic transmembrane (CRAM) protein which may be a lipoprotein receptor (Lee *et al.*, 1990) do contain cysteine residues. Metacyclics do, however, express VSG which like bloodstream VSG contain cysteine residues forming disulphide bridges to conserve the tertiary structure (Carrington and Boothroyd, 1996). Consequently the availability of cysteine in the midgut may affect the decision of the trypanosome to mature.

Cysteine proteinases are thought to play an important role in trypanosomes and their inhibition rapidly kills *T. b. brucei* (Troberg *et al.*, 1999). During differentiation from the long slender to the short stumpy there is a 10- to 15-fold increase in cysteine proteinase activity, however, very little activity could be detected in procyclic trypanosomes (Pamer *et al.*, 1989). Metacyclogenesis in *T. cruzi* is blocked by cysteine proteinase inhibitors (Bonaldi *et al.*, 1991) and there is stage specific expression of distinct cysteine proteinases in *Leishmania mexicana* (Souza *et al.*, 1992; Bates *et al.*, 1994) suggesting that cysteine proteinases play an important role in the differentiation of the Kinetoplastida.

GSH had no effect on transmission rates suggesting that it is not broken down into its component parts in the gut, which would release L-cysteine. Huynh *et al.* (2003) found that cultured trypanosomes could take up GSH from the culture medium; however, trypanosomes in the ectoperitrophic space may not have access to GSH. The inability of GSH to influence transmission rates also suggests that if GSH is taken up by trypanosomes then it is unlikely that the production of trypanothione is involved in maturation.

Uric acid has been implicated in enhancing the metacyclogenesis of *Trypanosoma musculi*; the addition of uric acid to cultures of *T. musculi* enhanced the production of metacyclic forms (Rogers and Viens, 1987). Although in the present work transmission was increased by continually feeding uric acid in males from 51% to 64%, the difference was not significant, while there was only a 1% difference in transmission rates in female flies. This suggests that uric acid plays no part in the metacyclogenesis of *T. b. brucei*.

Continual addition of ascorbic acid to the tsetse diet completely blocked maturation in both male and female flies. This was not due to a toxic effect of ascorbic acid as it promotes midgut infections and trypanosome levels within the midguts were not different from control flies. Recent work by Wu *et al.* (2003) has shown that ascorbic acid can inhibit inducible nitric oxide synthase (iNOS) and the present work has shown that the addition of L-NAME a nitric oxide synthase (NOS) inhibitor reduces maturation rates in both male and female tsetse. This suggests that nitric oxide (NO) may play a role in promoting maturation. Previous work had shown that D-glucosamine inhibits maturation by inhibiting tsetse lectins which were thought to provide a signal to the trypanosome to mature (Welburn and Maudlin, 1989). In recent years evidence has accumulated that glucosamine can inhibit iNOS and glucosamine salts are now widely used in the treatment of osteoarthritis, their anti-inflammatory properties operating through inhibition of the NO pathway (Shikhman *et al* 2001; Orth, *et al* 2002). In the present work addition of aminoguanidine, a NOS inhibitor, also blocked the maturation of *T. b. brucei*, but it also reduced the number of midgut infections to nearly half that of the control value. Although used as iNOS inhibitors, derivatives of aminoguanidine have been shown to have antitrypanosomal properties (Ross and Jamieson, 1975). Aminoguanidine can also interfere with advanced glycosylation end-product formation, diamine oxidase, polyamine metabolism and catalase (Alderton *et al.*, 2001), while Zhou *et al.* (2002) found that mice lacking iNOS were more susceptible to infection with *Salmonella typhimurium* when they had received aminoguanidine. This suggests that aminoguanidine may affect more than NO synthesis and the reduction in trypanosome infection rates may be caused by other actions of aminoguanidine. Although the use of two NOS inhibitors reduced maturation, the addition of L-arginine had no significant effect on maturation, suggesting that provision of the substrate of NO is not enough to promote maturation in its self. Further attempts during the current work to manipulate NO levels by continual addition of *E. coli* to the bloodmeal, a method shown to increase NO levels (Hao *et al.*, 2001), resulted in high numbers of tsetse deaths and reduced maturation rates (data not shown). Although the addition of 5 mM spermine-NO complex blocked maturation, 5 mM spermine on its own had the same

effect. However, the spermine-NO complex had a drastic effect on the resident parasite population and very low numbers of parasites were found (data not shown).

While NO is toxic at high concentrations it can be anti-apoptotic at lower levels (Fiscus, 2002). Low levels of NO oxide have been shown to be protective to human monocytes and induce differentiation to dendritic cells (Fernandez-Ruiz *et al.*, 2004). It is likely that if trypanosomes are responding to NO then the concentration would be crucial. The addition of an NO donor in the present work was obviously toxic to the trypanosomes while high levels produced in response to *E. coli* could also have had a detrimental effect on trypanosomes.

### **5.3.2 The effects of cyclic nucleotides on maturation of midgut infections of *T. b. brucei* in *G. m. morsitans***

Second messengers have been shown to be involved in control of differentiation of several cell types (Hagiwara *et al.*, 1996; Niedbala *et al.*, 2002; Saran *et al.*, 2002; Pilz and Casteel, 2003; Pukkila-Worley and Alspaugh, 2004). cAMP has been shown to be important in the transformation of long slender trypanosomes to short stumpy forms (Vassella *et al.*, 1997). However, provision of 8-Br-cAMP to the diet of the tsetse fly with established midgut infections had no effect on maturation rates in males or female tsetse when the infective feed contained either 100  $\mu$ M 8-Br-cGMP or 15 mM GSH. 8-Br-cGMP has been shown to promote midgut infections (see chapter 4.2.6), however, continually feeding 8-Br-cGMP to the fly had no effect on maturation rates when the infective feed contained 100  $\mu$ M 8-Br-cGMP. When the infective feed contained 15 mM GSH maturation rates were lower than controls, although this decrease in maturation rates was only significant when flies were dissected after 14 d. Flies which had received 100  $\mu$ M 8-Br-cGMP in the infective feed and dissected after 14 d rather than at 28 d produced low levels of transmission, with 3% in both those with 100  $\mu$ M 8-Br-cGMP in the infective feed and those continually fed 100  $\mu$ M 8-Br-cGMP. This suggests that

maturation may be delayed by 8-Br-cGMP but the barrier created is overcome 28 d post-infection.

As 8-Br-cGMP can increase midgut infection rates when injected into the haemolymph of the fly (chapter 4.2.11) it is unlikely that failure to promote maturation is due to lack of exposure to cyclic nucleotides, suggesting that factors other than cAMP or cGMP are required to promote maturation.

### **5.3.3 The effects of energy reserves on maturation of midgut infections of *T. b. brucei* in *G. m. morsitans***

The main energy substrate for both tsetse flies and trypanosomes is the amino acid proline (Bursell, 1981). To examine if the amount of energy available to the trypanosome played a role in maturation the fly diet was supplemented with 20 mM proline. No significant differences were found between control flies and those fed proline suggesting that the availability of an energy source does not influence the ability of a resident midgut infection to mature.

### **5.3.4 The effects of temperature on maturation of midgut infections of trypanosomes in *G. m. morsitans***

Temperature plays an important part in the trypanosome life cycle. During transfer from the mammal to the tsetse fly the trypanosome is subjected to a change in temperature from 37°C to 25°C and vice versa. *In vitro* the most important factor in differentiation of trypanosomes from bloodstream to procyclic form is a temperature drop from 37°C to 26°C (Bass and Wang, 1991). Previous experiments had shown that the temperature that tsetse flies were kept at was important in determining the maturation of midgut infections (Kinghorn and Yorke, 1913; Dipeolu and Adam, 1974; Otieno *et al.*, 1983). Otieno *et al.* (loc. cit) reported raising maturation rates in male tsetse which had been



chilled for 30 min after feeding on the infective bloodmeal. Re-examination of the data of Otieno *et al.* (loc. cit.) suggests that although the number of salivary gland infections increased (due to a significant increase in midgut infections), there was in fact no significant difference between the proportions of midgut infections maturing into salivary gland infections. During the current work, female tsetse that were chilled at 4°C for 30 min 4 d post-infection matured significantly more salivary gland infections than female flies that had not been chilled. The fact that a temperature shock did not increase rates of maturation in male flies, suggests that there is a natural limit to maturation indices that are normally reached in males, or that the factor/s which inhibit maturation in females are negated by chilling.

To investigate if cold shock quickened the maturation process of trypanosomes, infected female tsetse were dissected at 14 d or 21 d. Flies dissected after 21 d which had been chilled for 5 min or 1 h showed significantly increased transmission rates. Although chilling for 3 h increased transmission rates, this increase was not significant due low numbers of flies surviving to dissection. No significant difference was found between chilled and control flies of either sex dissected after 14 d showing that chilling does not increase the speed of maturation. These results suggest that cold shock breaks down the barrier to transmission in female flies which would not previously have matured an infection. Cold shock had no effect in either male or female *G. m. morsitans* infected with *T. b. rhodesiense* (strain DO), suggesting that maturation rates are also trypanosome strain dependent. As with experiments where the bloodmeal was continually supplemented with L-cysteine, no maturation at all occurred in female flies infected with DO.

During darkness in southern Africa temperatures can fall close to freezing (Phelps and Burrows, 1969). Fairburn (1948) found fewer cases of sleeping sickness during the colder part of the year suggesting that cold shock during darkness does not play a role in wild flies. However, incidence of sleeping sickness is unlikely to relate directly to environmental temperature given all the social variables affecting the epidemiology of the disease (Welburn *et al.*, 2004). When examining maturation rates in tsetse in the laboratory, flies are kept at a constant temperature; this would not be the case in the wild



where temperatures would fluctuate during the day. It would be interesting to see whether varying the temperature during the day had an effect maturation rates in tsetse that were infected with trypanosomes.

### **5.3.5 The effects of mating on maturation of midgut infections of *T. b. brucei* in *G. m. morsitans***

In the wild, female tsetse flies will usually be inseminated within a few days of emerging from the pupae (Hargrove, 1994). However, in laboratory experiments unmated females are usually used when investigating transmission rates of trypanosomes in tsetse. In the current work the effect of mating on maturation of trypanosomes was examined in female tsetse. Mated female tsetse flies produced significantly fewer trypanosome infections than unmated females, suggesting that mating or producing offspring has a detrimental effect on the maturation of trypanosome infections in female tsetse flies. This could be due to a reduction in the amounts of free nutrients available to trypanosomes, which instead go towards larval production in the female; levels of circulating hormones will also be different in pregnant tsetse. Moloo and Kutuza (1985) investigated the effect of trypanosome infection on the reproductive output of the tsetse fly. Flies fed on animals infected with *T. vivax*, *T. congolense* and *T. brucei* showed no significant differences between the number of pupae produced or pupal weight compared to control flies fed on uninfected animals, suggesting that a trypanosome infection does not affect the ability to produce offspring. However, in the wild, tsetse will need to find their own blood supply which may have an effect on pupal production if the tsetse is infected with trypanosomes (Bursell, 1981) Although levels of cyclic nucleotides fluctuate during pregnancy in tsetse (Denlinger *et al.*, 1984) and virgin females can be induced to ovulate in response to cAMP (Denlinger *et al.*, 1978), in the current work continuous feeding of cGMP and cAMP had no effect on maturation rates. This would suggest that it is the process of producing the offspring rather than ovulation that could reduce maturation in mated females. The large reduction

in maturation observed here (from 67% to 25%) suggests that mating more than halves the ability of the trypanosome to mature. However, all the flies used had midgut infections boosted due to the addition of GSH to the infective feed and maturation was increased by the cold shock needed to separate the flies. In the wild, most flies are refractory to infection with trypanosomes so will not have a midgut infection to mature. If a mated female in the wild is less likely to transmit an infection and male flies do not live long enough to transmit trypanosomes this could account for the small number of salivary gland positive flies found in the wild.

Although there have been reports on the vectorial capacity of sterile males (Moloo *et al.*, 1988) there have been no investigations on transmission of trypanosome infections in female flies which have been mated with sterile males. The present work has shown that unmated females produce twice as many mature infections as mated females, thereby a female in the wild which mates with a sterile male may be a better vector of pathogenic trypanosomes than a female which had mated with a wild type male. Given that the sterile insect technique (SIT) is going to be used as a frontline method to eradicate tsetse in Africa (Rogers and Randolph, 2002) it would be interesting to investigate whether females which had mated with sterile males were more efficient vectors of pathogenic trypanosomes than females fertilised by wild type males.

### **5.3.6 The sex of the tsetse and maturation of midgut infections**

Throughout the present work male tsetse matured greater proportions of midgut infections than female flies agreeing with previous work by Maudlin *et al.*, (1990) and Moloo *et al.*, (1992). Milligan *et al.* (1995) suggested that an X-linked gene(s) kills or otherwise prevents migrating parasites from establishing a mature infection. This female effect could be overcome by feeding female flies L-cysteine (TI=39%) which raised the maturation rates to levels produced in male flies (TI=37%) which were not fed cysteine. Although TI was lower in female flies (TI=48%) that were chilled when compared to male flies, there was no significant difference, between either control males (TI=58%) or

males that had been chilled (TI=62%). This suggests that both L-cysteine and a cold shock overcome the factor(s) which normally reduce maturation rates in female flies; however, since L-cysteine increased TI in male flies as well, it suggests that they represent two separate processes.

No maturation was observed in female flies infected with *T. b. rhodesiense* (stain DO), this was not due to the trypanosomes not possessing the ability to mature, as male flies did vector these infections successfully. Welburn *et al.* (1995) found that 27% of human serum resistant trypanosomes failed to mature an infection in female *G. m. morsitans* suggesting that DO, like other *T. b. rhodesiense* trypanosomes, lacks the ability to mature infections in female flies. Further experiments need to be undertaken to investigate the effects of cysteine and cold shock in other trypanosome stocks and tsetse species.

### 5.3.7 Conclusions

There has been little work published on maturation of procyclic populations resident in tsetse flies. The present work has shown that the addition of L-cysteine to the bloodmeal of tsetse infected with trypanosomes results in significant increases in maturation rates of both *T. b. brucei* and *T. b. rhodesiense* in *G. m. morsitans*. The same effect was not observed when D-cysteine was added to the infective bloodmeal suggesting that this is a non-specific antioxidant effect involving trypanosome synthesis of protein(s) containing L-cysteine.

Although L-arginine had no effect on transmission rates, it is intriguing that the NOS inhibitor L-NAME decreased maturation rates of midgut infections in both male and female flies, suggesting a role for NO in transmission. NO is known to exert the vast majority of its actions through cGMP and although continually feeding 8-Br-cGMP to tsetse had no significant effect on transmission rates, when the infective bloodmeal was supplemented with GSH there was a decrease in maturation, a difference which only

became apparent after 14 d. This suggests that if NO is involved in maturation then the effect is independent of cGMP at least after 28 d.

Ascorbic acid was the only compound tested that acted like glucosamine (increasing midgut infections while lowering TI). However, the concentration of ascorbic acid required to achieve transmission blocking was very high (20 mM) while lower concentrations (1 mM) had no effect on maturation. As serum levels of ascorbic acid in mammalian hosts are low [e.g. in humans: 20-90  $\mu$ M (Halliwell, 2001)] it is unlikely that under normal conditions, serum levels of ascorbic acid would lead to a decrease in transmission rates and while artificially increasing serum levels of ascorbic acid concentrations to levels which could influence transmission rates it is likely that it could lead to toxic effects on the mammal. There is little information on ascorbic acid levels in trypanosome infected host animals.

The triggers for maturation of trypanosome infection in tsetse are still poorly understood but with both L-cysteine and cold shock significantly increasing transmission rates in female flies this could provide the material for isolation of specific mRNAs involved in the maturation process.

## **Chapter 6**

### **General discussion**

## 6.1 The effects of symbionts on trypanosome infections

This thesis has investigated factors which may affect the outcome of an encounter between a tsetse and a trypanosome. It is obvious from studies on trypanosome infection rates that instances of mature infections within tsetse flies are rare (Okoth and Kapaata, 1986) and laboratory studies have shown that a high percentage of flies are refractory to midgut infection (Hao *et al.*, 2001). Susceptibility to trypanosome infection was linked to the maternally inherited bacterium *S. glossinidius* (Maudlin *et al.*, 1986) and a model was drawn up suggesting that these bacteria produced sugars during pupal development which inhibited trypanocidal lectins secreted during the bloodmeal (Welburn *et al.*, 1993). The fact that *S. glossinidius* was detected by PCR in all colony tsetse flies examined and with the same colony producing low trypanosome infection rates suggests that possession of *S. glossinidius* will not in itself make tsetse flies more susceptible to infection. Indeed recent work has shown that the DNA probe used in these studies (Maudlin *et al.*, 1990; Welburn and Maudlin, 1991) was found to be specific to a bacteriophage present in *S. glossinidius* (Ian Maudlin, personal communication), suggesting that it is possession of phage rather than *S. glossinidius* which is important in susceptibility to trypanosome infection. In the current work attempts were made to create a line of flies which did not possess *S. glossinidius*; however, due to several factors, most notably the low fecundity of tsetse treated with streptozotocin, this work was discontinued. Given that the refractory line set up by Maudlin (1982) slowly regained susceptibility to trypanosome infection (Welburn and Maudlin, 1991), this suggests that the factor(s) which conveyed susceptibility returned to the refractory colony. Alternatively if a phage was involved in susceptibility to infection, then a refractory tsetse may still contain a low number of phage, which although not able to influence the susceptible status of its current host, in future generations phage numbers may increase and over several generations the flies derived from this tsetse could show increased levels of susceptibility. This would suggest that if another attempt was made

to set up a tsetse colony deficient in *S. glossinidius* then it should be kept completely isolated from the colony from which it was derived.

## 6.2 Midgut infections of trypanosomes

Tsetse flies possess a potent system which efficiently kills trypanosomes ingested with the bloodmeal. Previous work had shown that addition of glucosamine (Maudlin and Welburn, 1987) or removal of serum (Maudlin *et al.*, 1984) from the bloodmeal could increase midgut infection rates and the current work has shown that these responses can also be circumvented by the addition of a variety of antioxidants or cGMP to the bloodmeal. It is interesting that both L- and D-cysteine invoke the same trypanosome midgut infection rates, ruling out the possibility that synthesis of proteins is involved and instead suggesting that cysteine acts directly by detoxifying the midgut environment that the trypanosomes must circumnavigate before they can establish an infection in the ectoperitrophic space. The fact that ascorbic acid and uric acid also increased susceptibility to trypanosome infection rules out a thiol specific response as glutathione (GSH), N-acetyl-cysteine (NAC) and cysteine all contain a sulfhydryl group. The time of exposure to GSH was found to be crucial, addition of GSH to either the infective bloodmeal or 2 d post-infection significantly increased midgut infection rates, however, at 3 d post-infection GSH had no significant effect. This suggests that the trypanosomes had entered an apoptotic state which GSH could not block, sometime between 2 d and 3 d post-infection. However, 8-Br-cGMP could still significantly increase trypanosome infection rates 4 d post infection, suggesting that the trypanosomes could still be rescued from this apoptotic state. Given that the exposure to *E. coli* when either fed or injected into the fly resulted in the lowering of trypanosome midgut infection rates, this suggests that the bacteria invoke a response in the fly which is trypanocidal. Previous experiments had showed that attacin (Hao *et al.*, 2001) and nitric oxide (NO; Hao *et al.*, 2003) levels are raised in response to *E. coli*. However, the increased levels of attacin mRNA synthesis were observed in the fat body and although attacin has been found in



the haemolymph of tsetse flies (Kaaya *et al.*, 1987) and is also synthesised by the proventriculus (Hao *et al.*, 2001), it is unclear if attacin is secreted into the gut of the tsetse. Sun and Faye (1995) found that attacin mRNA levels were reduced in the giant silkworm *Hyalophora cecropia* when thiols were administered both *in vitro* and *in vivo*. In the present work injection of NAC or GSH into the haemolymph of tsetse failed to affect infection rates and it would seem that antioxidants in the haemolymph would not directly affect the synthesis of immune peptides in the fat body or proventriculus. However, if hydrogen peroxide signalling from the gut was involved in the synthesis of attacin in the fat body, then the addition of antioxidants to the bloodmeal could disrupt this pathway. Future work could investigate if the addition of antioxidants or cGMP affected synthesis of attacin or other immune peptides and if attacin was secreted into the midgut where the trypanosomes reside.

Recent work in this laboratory has shown that procyclic trypanosomes incubated with 8-Br-cGMP showed differential gene expression, inducing changes in the levels of mRNA involved in the synthesis of enzymes concerned with the formation of polyamines. Surface enhanced laser desorption ionization mass spectrometry (SELDI) analysis showed a strong down-regulation of a 28.5 kDa protein and up-regulation of two proteins 33-34 kDa (Hachemi Zeraia, personal communication). This could suggest that cGMP provides a signal to the trypanosome to start producing substrates of trypanothione or other compounds which could override the apoptosis process. The production of trypanothione would effectively reduce oxidative stress on the parasite and allow colonisation of the tsetse gut. This would fit in with the experiments described here involving NAC; 10 fold less NAC was required to give similar infection rates when compared to GSH or cysteine. The cell permeable NAC could easily cross the trypanosome membrane and reduce oxidative stress in the same way as the synthesis of trypanothione would in the trypanosome itself. It is possible that in a fly susceptible to trypanosome infection the parasite receives a signal that results in the synthesis of trypanothione which could detoxify free radicals produced during bloodmeal breakdown. Recent work has shown that a protein kinase A in *T. brucei* functions

through cGMP (Shalaby *et al.*, 2001), suggesting that cGMP could play an important signalling role in trypanosomes.

The fact that neither ascorbic acid nor uric acid had any effect on midgut infection rates in *G. p. palpalis* suggests that differences exist in the gut environment of these riverine flies. Further experiments could investigate other tsetse species to examine the effect of the various antioxidants and whether ascorbic acid or uric acid increase susceptibility to trypanosome infection in other members of the *palpalis* group.

### 6.3 Maturation of established trypanosome midgut infections

Mature infections of *Trypanozoon* within tsetse flies in the wild are notoriously rare (Okoth and Kapaata, 1986). This is the same in the laboratory and although it is possible to create lines of flies which have a higher susceptibility to initial midgut infections it has so far not been possible to create a similar line of flies showing increased transmissibility of midgut infections (Ian Maudlin, personal communication). This, however, may be due to the fact that it is trypanosome rather than fly factors or a combination of the two that are responsible for maturation. Previous work had postulated that trypanosomes possessed lectin binding sites and that longer exposure to midgut lectins increased the frequency of maturation (Welburn and Maudlin, 1989). Although there is still no direct evidence implicating trypanosome or fly factors in maturation, the fact that L-cysteine but not cystine can increase maturation rates [dipterans can utilise cystine (Hazelton and Lang, 1983), while trypanosomes cannot (Duszenko *et al.*, 1992)], suggests that it is in fact the trypanosome which is responsible for maturation; since D-cysteine had no effect, this suggests the synthesis of cysteine containing protein(s) are involved in the maturation process. Further work examining protein synthesis in both the trypanosome and the tsetse in response to provision of L-cysteine could solve this problem. However, one of the main factors pointing to tsetse control of maturation is the difference seen between male and female flies with *Trypanozoon* infections; males mature more midgut infections than females (Welburn *et*

*al.*, 1995). The current work has shown that chilling of infected female, but not male tsetse leads to increased transmission rates suggesting that it is a fly factor which reduces maturation in females, which is somehow overcome by cold shock. As addition of L-cysteine to the bloodmeal or chilling of infected female flies leads to increased salivary gland infections it would appear that there are at least two distinct processes involved in maturation. However, temperature stress can change GSH levels; for example the response to cold shock in *E. coli* is a reduction in the intracellular content of GSH when grown at 20°C (Smirnova, 2001). This could lead to the increased levels of L-cysteine due to the decreased synthesis of GSH. However, if increased levels of cysteine are important in trypanosome maturation it could still be a fly factor that either provides the cysteine or gives a signal to the trypanosome to synthesise cysteine; since male flies mature more midgut infections than females this suggests that there is a fly factor involved as there was no increase in maturation rates in males as a consequence of cold shock.

In the current work maturation rates were examined only in *G. m. morsitans*; future work could investigate whether other tsetse species responded in the same way. Since trypanosome midgut infections rates were unaffected in *G. p. palpalis* by the addition of ascorbic acid, it would be interesting to see if the compounds tested here had any effect on maturation rates of trypanosomes in other species of tsetse.

## 6.4 Outlook

It is just over 100 years since Bruce made the link between trypanosome transmission and the tsetse fly. Since then several attempts have been made to eradicate tsetse from Africa; game destruction and most notably ground spraying of insecticides such as DDT rendered many African areas tsetse free. However, during the past 40 years sleeping sickness and the tsetse fly have been making a comeback mainly due to the reduction of tsetse control programs. The present work has shown that the establishment of midgut infections can be increased by either antioxidants or cGMP and although one antioxidant

increased maturation rates, others decreased them or had no effect. Investigating how ascorbic acid and L-NAME reduced maturation rates could result in the production of transmission blocking agents which could decrease the transmissibility of trypanosomes through tsetse flies.

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## Appendix 1

The methods from Otieno *et al.* (1983) were as follows; Emerging *G. m. morsitans* were collected in polyvinyl glue cages at one or two hourly intervals. The various groups of young flies collected were exposed to an infective bloodmeal 22-24, 20-22, 18-20, 16-18, 14-16, 12-14, 7-8, 6-7, 5-6, 4-5, 3-4, 2-3 and 1-2 h after emergence. As soon as these flies had fed, one half of each age group was transferred to a refrigerator at 0.5°C (for 30 minutes) and the other half kept at 25°C and 80% relative humidity. After the short cold exposure all flies were transferred to the insectary at a temperature of 25°C and maintained on two clean rabbits. The flies were dissected and examined for the presence of trypanosome infection 20 days after the infective bloodmeal. The results from Otieno *et al.* (1983) are presented Table appl.

Cooled flies					Control flies				
time	No.	Gut infections	Salivary gland infections	Salivary gland infections %	time	No.	Gut infections	Salivary gland infections	Salivary gland infections %
22-24	15	3	1	7%	22-24	15	1	1	7%
20-22	13	4	2	15%	20-22	15	1	1	7%
18-20	15	4	2	13%	18-20	14	4	1	7%
16-18	19	7	5	26%	16-18	14	3	1	7%
14-16	29	4	3	10%	14-16	23	5	3	13%
12-14	2	0	0	0%	12-14	3	0	0	0%

Total	93	22	13	14%		84	14	7	8%
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7-8	11	7	5	45%	7-8	10	3	2	20%
6-7	16	7	6	38%	6-7	15	8	2	13%
5-6	10	5	1	10%	5-6	11	3	2	18%
4-5	17	11	5	29%	4-5	16	4	2	13%
3-4	20	5	5	25%	3-4	16	7	3	19%
2-3	13	4	2	15%	2-3	12	0	0	0%
1-2	16	3	2	13%	1-2	19	3	2	11%

Total	103	42	26	25%		99	28	13	13%
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**Table app1 Data from Otieno *et al.* (1983). Legend: Cooled flies: flies chilled at 4°C for 30 min after bloodmeal ingestion; control flies: control flies not chilled after bloodmeal ingestion; time: time infected after emergence from the puparium; No.: number of flies dissected; Gut infections: number of midguts positive for trypanosomes; Salivary gland infections: number of salivary glands positive for trypanosomes; Salivary gland infections %: percentage of flies with salivary gland infections using the number of flies dissected as denominator.**